0021-972X/86/6305-1193$02.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 63, No. 5
Printed in U.S.A.

Mechanism of Inhibition of Human Testicular Steroidogenesis by Oral Ketoconazole*

JACOB RAJFER, SURESH C. SIKKA, FABIO RIVERA, AND DAVID J. HANDELSMAN†

Division of Urology, Department of Surgery, UCLA School of Medicine, Harbor-UCLA Medical Center, Torrance, California 90509

ABSTRACT. To determine the antisteroidogenic effect of ketoconazole (KTZ) in the human testis, we measured the plasma Δ^5-pregnenolone, Δ^5-17α-hydroxypregnenolone, dehydroepiandrosterone (DHEA), progesterone, 17α-hydroxyprogesterone, androstenedione (A), and testosterone (T) concentrations in three men with previously untreated metastatic prostate cancer at various time intervals for 24 h before and 48 h after the administration of 200 mg oral KTZ every 8 h. The adrenal glands of these three patients were suppressed (as measured by the plasma cortisol levels) by the administration of 1.0 mg dexamethasone daily for 7 days before and during the study. After six doses of KTZ, bilateral orchiectomy was performed, and the intratesticular concentration of the aforementioned seven steroids and the intratesticular activities of the 17α-hydroxylase, 17,20-desmolase, and 17β-hydroxysteroid dehydrogenase enzymes in the Δ^4-steroidogenic pathway were determined. These seven intratesticular steroids and three intratesticular enzyme activities were compared to those in five men with previously untreated prostate cancer who underwent orchiectomy as primary treatment for their disease. Plasma A, DHEA, and T all significantly decreased during KTZ therapy. There was no significant change in the other four steroids in the plasma. In the testis, Δ^5-pregnenolone, Δ^5-17α-hydroxypregnenolone, and Δ^4-17α-hydroxyprogesterone were all significantly elevated, whereas intratesticular DHEA, A, and T were significantly decreased in the three KTZ-treated patients compared to levels in the five non-KTZ-treated patients. Measurement of the enzyme activities demonstrated a significant reduction in both 17α-hydroxylase and 17,20-desmolase, but no change in 17β-hydroxysteroid dehydrogenase, in the KTZ-treated patients compared to the levels in the non-KTZ-treated patients. We conclude that oral KTZ decreases testicular T production by inhibiting the 17,20-desmolase and also the 17α-hydroxylase steps in both the Δ^4- and Δ^5-T biosynthetic pathways. (*J Clin Endocrinol Metab* **63:** 1193, 1986)

KETOCONAZOLE (KTZ) is a synthetic, orally active imidazole dioxolone derivative introduced initially as a broad spectrum antifungal agent (1). More recently, KTZ was found to be a potent inhibitor of human gonadal (2) and adrenal (3, 4) steroidogenesis. A common mechanism of action of KTZ has been proposed, since inhibition of a wide variety of cytochrome P-450-dependent enzymes by KTZ has been described in fungal membranes (5) and adrenal steroid production (4). Steroidogenesis in the testis, however, is much more sensitive to inhibition by KTZ compared to that in the adrenal, since production of testicular C-19 steroids is profoundly inhibited by KTZ at doses that do not inhibit basal production of cortisol and other C-21 adrenal steroids (2, 3). Thus, it may be hypothesized that a major site of steroidogenic blockade by KTZ in the testis is 17,20-desmolase, a cytochrome P-450-dependent enzyme that catalyzes conversion of C-21 steroidal precursors to C-19 sex steroids.

The inaccessibility of human steroidogenic tissues has limited the ability to test this hypothesis directly in men treated with KTZ. Previous studies attempted to infer the site of action of KTZ by examining the profiles of circulating steroid precursors (2, 3, 6). This indirect approach has several difficulties, including the dual adrenal and gonadal sources of such steroid precursors, alterations in steroid clearance rates, and the introduction of additional intermediate variables, such as possible drug effects on circulating steroid-binding protein levels or binding affinity. An alternative approach of studying the effects of KTZ on steroidogenesis *in vitro* (7) entails some difficulties in interpretation, since the possible effects of active metabolites of KTZ cannot be adequately addressed. In this study we examined the site(s) whereby KTZ inhibits testicular testosterone (T) production in man by direct measurement of testicular steroidogenic enzyme activities after *in vivo* treatment. In addition, to

Received March 10, 1986.
Address requests for reprints to: Jacob Rajfer, M.D., Harbor-UCLA Medical Center, 1000 West Carson Street, Box 5, Torrance, California 90509.
* This work was supported in part by a grant from the NIH (GCRC RR-00425).
† National Health Medical Research Council Overseas Fellow (Australia). Current address: Department of Medicine, University of Sydney, Sydney, 2006, New South Wales, Australia.

further define the relationship between the site of steroidogenic inhibition and circulating steroid precursors, we simultaneously measured both testicular and serum steroid levels in KTZ-treated men who underwent concurrent adrenal suppression. Our results indicate that KTZ inhibits testicular steroidogenesis primarily by inhibiting 17,20-desmolase activity.

Materials and Methods

Patients

Three unselected men (mean age, 69 yr; range, 63–76 yr) with newly diagnosed metastatic (stage D) prostate cancer who were about to undergo bilateral orchiectomy as the conventional primary treatment for their disease were enrolled into this study. Before entry into the study, each man gave written informed consent to undergo blood sampling and dexamethasone (DEX) and KTZ treatment before orchiectomy. All protocols and procedures were approved by the Human Subjects Ethical Review Committee of the Harbor-UCLA Medical Center. Testes excised from five other unselected men (mean age, 69 yr; range, 63–82 yr) with (stage D) metastatic prostate cancer who had received no prior drug treatment were used as controls for measurement of testicular steroidogenic activity. None of the patients in the control or KTZ-treated groups had received any prior hormonal therapy for their disease.

Protocol

The men commenced taking oral DEX (0.5 mg daily) at least 7 days before admission to the Clinical Study Center, Harbor-UCLA Medical Center, and DEX was continued until surgery 3 days after admission. After admission, a venous cannula was inserted into a forearm vein, and aliquots of blood were withdrawn at various time intervals for the next 72 h until orchiectomy. During the control day (first 24 h after admission), blood was sampled at −24, −20, −16, −8, −4, −2, −1, and 0 h. At the start of the second day and for the following 48 h until surgery, 200 mg KTZ (Janssen Pharmaceutica, Beerse, Belgium) were administered orally every 8 h in addition to DEX, and blood was sampled at 1, 2, 4, 8, 12, 16, 20, 24, 32, 40, and 48 h. The blood was allowed to clot, and the serum was separated and stored at −20 C until performance of steroid assays.

At surgery, both testes were removed, snap-frozen in liquid nitrogen, and stored at −70 C until processing for measurement of testicular steroidogenic enzymes and steroid levels.

Measurement of steroid levels and enzyme activities

The testes from both the control (untreated) and KTZ-treated groups were quickly thawed, decapsulated, and homogenized at 4 C using a motor-driven glass homogenizer by 15–20 strokes at about 2000 rpm in ice-cold 0.1 M phosphate buffer, pH 7.4 (1:5, wt/vol), containing 0.25 M sucrose and 1 mM dithiothreitol. The homogenate was centrifuged at 800 × *g* for 20 min, and the supernatant was either used immediately for the enzyme assays or kept at −70 C until used for the determination of steroid concentrations and enzyme activities.

The steroid concentrations in testicular homogenates and serum samples were measured using RIA procedures, as described by Abraham *et al.* (8). An aliquot (1.0 ml) of homogenate (equivalent to ~200 mg tissue) or 1.0 ml serum was used for extraction with diethyl ether after trace amounts (~1000 cpm) of various steroids were added to determine their recoveries. Steroids were separated in batches by Celite column chromatography techniques, as described by Manlimos and Abraham (9). RIA procedures were carried out using purified tritiated tracers, authentic standards, and specific antibody preparations (RSL, Inc., Carson, CA). These specific antibodies were raised employing steroid-carboxyether-BSA or steroid-hemisuccinate-human serum albumin conjugates. The assays were performed after making appropriate dilutions of the recovered steroid samples. Dextran-coated charcoal was used to separate the free from the bound radiolabeled steroids. The specificity and accuracy of the assays were determined by assaying different volumes of the extracts as well as by monitoring the recovery of the added steroids. The recovery of the added steroids varied between 90–110%, and no systematic deviation of results from linearity was found. The cross-reactivity data showed that only dihydrotestosterone cross-reacted (~20%) in the T assay; for the other steroid assays, the cross-reactivities of major steroids were less than 1%. KTZ, in amounts up to 40 μg, did not cross-react with the T antibody. The sensitivity of the assay procedures was 20 pg for Δ^5-pregnenolone (PG),[1] 15 pg for progesterone (P), 10 pg for 17α-hydroxyprogesterone (17-P), 30 pg for androstenedione (A), and 10 pg for T. The intraassay variations for these steroids were 9% for PG, 7% for P, 8% for 17-P, 10% for A, and 7% for T, while the interassay coefficient of variation varied from 10–18%. Serum cortisol levels were determined, as previously described (10), employing [^{125}I]cortisol and anticortisol antibody (RSL, Inc.). All samples from an individual patient were analyzed at the same time.

Measurements of 17α-hydroxylase, 17,20-desmolase, and 17β-hydroxysteroid dehydrogenase activities in the Δ^4-steroidogenic pathway were carried out as previously described (11).

Statistical analysis

To optimize analytical design of this intensive study (19 samples/subject) of a small number of subjects (n = 3 subjects) and to account for baseline differences between subjects, serial measurements of serum steroids within individual patients were analyzed by repeated measures analysis of variance which considered each subject as his own control. Where appropriate, log transformations of hormone levels were used before analysis to ensure homogeneity of variance. The effects of KTZ during specified time periods were analyzed with F tests on suitable 1 degree of freedom linear contrasts. Analyses of variance were performed using the BMDP program 4V implemented on a VAX 11/750 computer (12). Intratesticular steroids and steroidogenic enzyme activities were compared between KTZ-

[1] The following trivial names are used. Δ^5-pregnenolone (PG), 5-pregnen-3β-ol-one; 17α-hydroxyprogesterone (17-P), 4-pregnen-17α-ol-3,20-dione; 17α-hydroxypregnenolone (17-PG), 5-pregnen-3β-17α-diol-20-one; dehydroepiandrosterone (DHEA), 5-androsten-3β-ol-17-one; androstenedione (A), 4-androsten-3,17-dione.

treated and control men by unpaired t test. Statistical significance was set at a 2-tailed α level of 0.05.

Results

The KTZ-treated and control groups were similar in age and stage of prostate cancer. No side-effects (hepatotoxicity) occurred during KTZ treatment and up to 1 month after orchiectomy.

Serum steroids

Serum cortisol levels in the DEX-treated men were suppressed (Table 1), indicating virtually complete suppression of glucocorticoid output during the entire study. This degree of adrenal suppression suggested that further changes in serum steroid levels could be attributed to changes in testicular, not adrenal, steroidogenesis. The low levels of serum cortisol before the initiation of KTZ therapy suggests compliance with DEX therapy.

By comparison with the 24 h of treatment with DEX alone, the addition of KTZ (600 mg/day) produced significant suppression (Table 1) of serum T ($F = 101.2$; $P = 0.01$), A ($F = 229.4$; $P = 0.004$), and dehydroepiandrosterone (DHEA; $F = 69.3$; $P = 0.014$), a marginal increase in 17-P ($F = 4.9$; $P = 0.16$), but no change in PG ($F = 0$; $P = 0.96$), 17-PG ($F = 0.01$; $P = 0.95$), or P ($F = 0.01$; $P = 0.93$). The decreases in serum T and DHEA were maximal by the end of the first treatment day, whereas A levels decreased further on the second treatment day ($F = 41.9$; $P = 0.023$). Serum 17-P increased significantly on the first day only ($F = 20.4$; $P = 0.041$). Diurnal effects during the 3 days of study were evident in serum T ($F = 22.8$; $P = 0.041$), DHEA ($F = 81.5$; $P = 0.012$), A ($F = 20.4$; $P = 0.046$), and 17-PG ($F = 332.3$; $P = 0.003$), but not PG ($F = 10.3$; $P = 0.085$), 17-P ($F = 7.6$; $P = 0.11$), or P ($F = 4.0$; $P = 0.184$; Table 1 and Fig. 1).

Intratesticular steroids

There was a significant ($P < 0.025$) decrease (75% lower) in intratesticular T levels, from 661 ± 155 (± SEM) ng/g testis in control patients to 163 ± 101 in KTZ-treated patients (Fig. 2). Intratesticular A levels decreased 75% ($P < 0.05$), from 139 ± 52 ng/g testis in control patients to 33 ± 12 in KTZ-treated patients. DHEA levels declined 43%, from 410 ± 46 ng/g testis in control patients to 232 ± 72 in KTZ-treated patients ($P < 0.05$). Intratesticular 17-P increased 1383%, from 12 ± 2 ng/g testis in control patients to 178 ± 82 in KTZ-treated patients ($P < 0.05$), while PG and 17-PG both increased 248% and 400%, respectively, in the KTZ-treated groups compared to levels in control patients. There was no difference in the intratesticular P content in the control and KTZ-treated patients. These results indicate that KTZ blocks primarily the 17,20-desmolase enzyme of both the Δ^4- and Δ^5-pathways of steroidogenesis.

Testicular steroidogenic enzyme activities

Testicular 17,20-desmolase activity (picomoles per min/mg protein) decreased 56%, from 590 ± 105 (± SEM) in control patients to 260 ± 20 in KTZ-treated patients ($P < 0.025$), while 17α-hydroxylase activity decreased 29%, from 1040 ± 135 in control patients to 740 ± 62 in KTZ-treated patients ($P < 0.05$). 17β-Hydroxysteroid dehydrogenase activity increased by 73% ($P < 0.05$) in the testes of KTZ-treated men (956 ± 292) compared to that in control men (554 ± 122; Fig. 2). These results are consistent with the inferences from product/precursor

TABLE 1. Effect of KTZ on serum steroid levels in DEX-treated prostate cancer patients

Steroids	Basal							KTZ[a]											
	−24 h	−20 h	−16 h	8 h	−4 h	−2 h	−1 h	0[a]	+1 h	+2 h	+4 h	+8 h[a]	+12 h	+16 h[a]	+20 h	+24 h[a]	+32 h[a]	+40 h[a]	+48 h[a]
PG (ng/dl)	182 ±14	165 ±23	137 ±18	112 ±18	148 ±28	168 ±14	176 ±52	177 ±79	185 ±32	213 ±19	214 ±62	193 ±32	210 ±43	150 ±6	166 ±14	250 ±16	140 ±41	154 ±20	180 ±12
17-PG (ng/dl)	42 ±17	27 ±7	18 ±3	41 ±14	33 ±5	28 ±8	38 ±8	46 ±4	47 ±18	39 ±10	42 ±12	4 ±7	35 ±13	33 ±16	3 ±19	44 ±28	3 ±22	41 ±28	37 ±25
DHEA (ng/dl)	50 ±8	30 ±2	23 ±3	34 ±10	3 ±5	33 ±6	47 ±12	46 ±7	42 ±7	24 ±3	21 ±5	23 ±2	19 ±4	15 ±2	15 ±1	11 ±3	10 ±4	16 ±1	11 ±5
P (ng/dl)	132 ±26	122 ±14	121 ±14	139 ±25	115 ±17	142 ±26	164 ±32	150 ±36	161 ±33	151 ±42	132 ±34	53 ±50	15 ±48	153 ±53	144 ±49	142 ±41	139 ±38	130 ±41	133 ±35
17-P (ng/dl)	66 ±14	44 ±15	45 ±17	62 ±18	76 ±27	61 ±22	66 ±21	49 ±17	57 ±10	66 ±21	71 ±10	83 ±21	95 ±18	82 ±14	85 ±35	84 ±28	104 ±34	127 ±50	77 ±17
A (ng/dl)	69 ±10	52 ±4	50 ±6	48 ±9	46 ±8	49 ±14	57 ±10	54 ±11	57 ±8	49 ±8	44 ±5	42 ±6	35 ±7	35 ±9	34 ±13	23 ±7	19 ±5	30 ±11	23 ±5
T (ng/dl)	708 ±351	476 ±148	503 ±219	631 ±231	668 ±242	644 ±252	610 ±217	586 ±176	549 ±156	408 ±176	232 ±90	338 ±132	279 ±119	295 ±134	155 ±65	219 ±62	134 ±21	265 ±40	203 ±60
Cortisol (ng/dl)[b]	450 ±190	ND	ND	240 ±43	226 ±26	ND	ND	496 ±29	ND	290 ±90	393 ±190	270 ±70	ND	270 ±70	ND	<200	<200	<200	<200

ND, not done. Values are the mean ± SEM.

[a] 200 mg KTZ, orally every 8 h.

[b] Normal 0800 h values, 10,000–25,000 ng/dl; all men (n = 3) received DEX (0.5 mg daily) throughout the study, starting 3 days before initiation of KTZ administration.

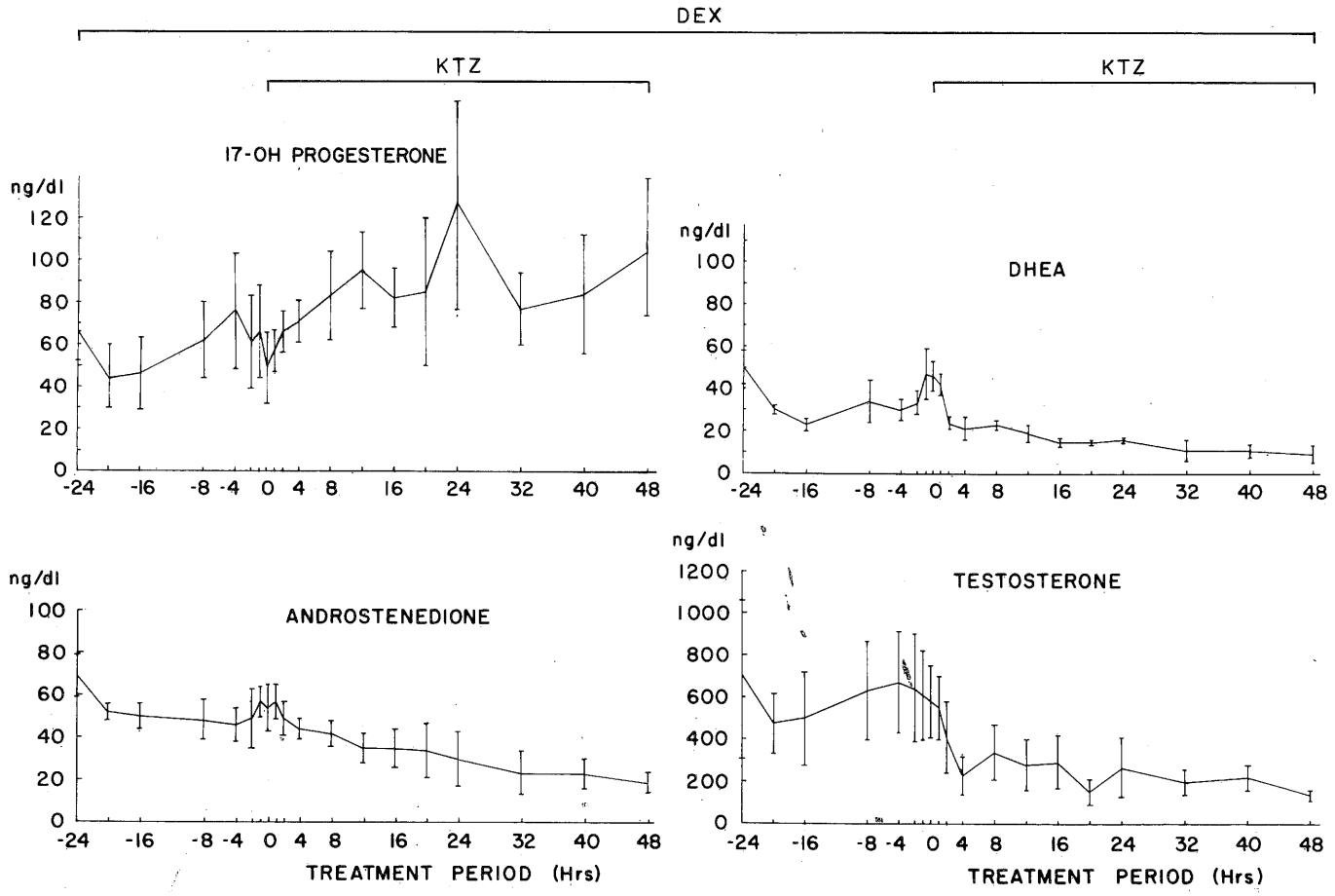

FIG. 1. Mean (±SEM) serum steroid levels (nanograms per dl) in three patients who received 3 days of KTZ therapy (200 mg, three times daily) and DEX (1.0 mg/day) during the 3 days of KTZ therapy and for 7 days before KTZ therapy.

ratios of steroids in either serum or testis, and again suggest that the principal site wherein KTZ inhibits T biosynthesis is the 17,20-desmolase enzyme.

Discussion

These results demonstrate clearly that KTZ inhibits testicular steroidogenesis by inhibiting 17,20-desmolase activity, a cytochrome P-450-dependent enzyme. This conclusion is supported by the concordance of the results of direct assays of testicular steroidogenic enzyme activities from KTZ-treated men and the contents of steroid precursors in the testis. For example, 17,20-desmolase activity was lower by nearly 60% in testes from KTZ-treated men compared to a 29% decrease in 17α-hydroxylase activity and a 73% increase in 17β-hydroxysteroid dehydrogenase activity. Similarly, testicular concentrations of the postdesmolase products (T, A, and DHEA) were all decreased, whereas the predesmolase precursors (PG, 17-PG, and 17-P) were increased. Whereas the pattern of precursor accumulation and product deficit was clearly evident in the testicular steroid concentrations, the steroid precursor patterns in serum were less clear-cut, despite the effective concurrent suppression of adrenal steroidogenesis. These results highlight the difficulty in making inferences about steroidogenic enzyme activities from concentrations of circulating steroids that have dual glandular origins and differing MCRs. The direct confirmation of KTZ inhibition of the 17,20-desmolase step *in vivo* effectively minimizes the importance of other potential KTZ-induced effects on circulating steroid-binding proteins or metabolic disposition of steroids.

This predominant site of KTZ inhibition may explain the greater sensitivity of testicular compared with adrenal steroidogenesis to KTZ (2, 3). The basal secretion of T is profoundly reduced by doses of KTZ that have no effect on basal cortisol secretion (2, 3). Theoretically, pure inhibition of the 17,20-desmolase step would interfere only with the production of C-19 steroids, thus altering secretion of sex steroids (C-19 androgens and estrogens), but not adrenal (C-21) glucocorticoids and mineralocorticoids. This is most closely exemplified by congenital 17,20-desmolase deficiency, which is associated with genital ambiguity due to abnormal C-19 steroid production whereas adrenal function is normal (13).

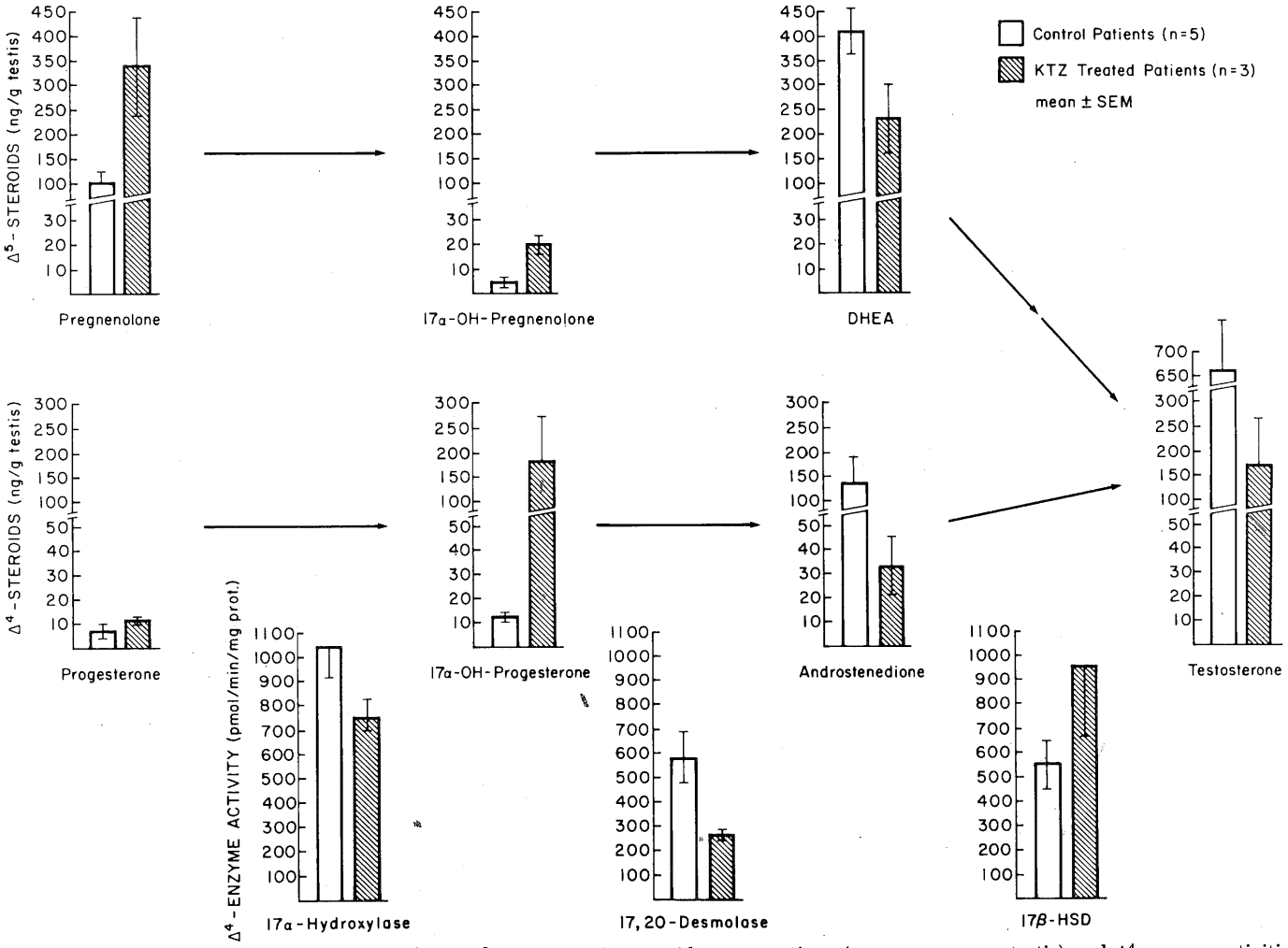

FIG. 2. Comparison between mean (±SEM) Δ^4 and Δ^5 intratesticular steroid concentrations (nanograms per g testis) and Δ^4 enzyme activities (picomoles per min/mg protein) in three patients who received KTZ and in five control patients. Note that enzyme activities are aligned to reflect their appropriate relationship to their respective substrates and products. See *Materials and Methods* for details.

The possibility of a second site of inhibition of KTZ is difficult to exclude from the data presented. Indeed, the general effects of KTZ on cytochrome P-450 enzymes (4) and their involvement in multiple steps in the steroidogenic pathways (13, 14) indicate that additional sites of action might be expected, albeit at higher doses reflecting the relative selectivity of KTZ on the 17,20-desmolase step. The lesser but significant inhibition of 17α-hydroxylase is consistent with a second site of action for KTZ, although recent evidence (15) confirmed the earlier hypothesis of Nakajin *et al.* (16) of the coexistence of 17α-hydroxylase and 17,20-desmolase in a single multifunctional enzyme complex in pig testis. Thus, inhibition of both enzymes in the human testis by KTZ might be attributable to a single site of action of KTZ on the multienzyme complex. The ability of KTZ to inhibit other adrenal cytochrome P-450 enzymes *in vitro* (4) and ACTH-stimulated adrenal reserve (3) *in vivo* suggests that at a sufficiently high dose, KTZ can inhibit other cytochrome P-450 enzymes, including the cholesterol side-chain cleavage (20,22-desmolase) and 11α-hydroxylase enzymes.

The pattern of enzyme inhibition demonstrated in this study is most directly applicable to the Δ^4-pathway, since the *in vitro* enzyme assays used Δ^4-substrates. Nevertheless, the consequences of 17,20-desmolase inhibition by KTZ were also clearly apparent in the accumulation in the testis of its Δ^5-substrate, 17-PG, and in reduction of its product, DHEA, consistent with the suggestion that a single or very similar enzymes catalyze each of the equivalent steps in the Δ^4- and Δ^5-pathways (12–16). The lack of a uniform increase in steroid precursors may be due to secondary sites of action of KTZ, differences in the sensitivity of various enzymes for KTZ effects, or a combination of these effects with the removal of feedback inhibition of the enzymes. The failure of both Δ^4- and Δ^5-precursors to accumulate to an equivalent degree in serum compared to their accumulation in the testis may

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.