Ketoconazole Blocks Adrenal Steroidogenesis by Inhibiting Cytochrome P450-dependent Enzymes

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ABSTRACT Ketoconazole has recently been shown to interfere with steroidogenesis in patients and rat in vitro systems. In this study we attempted to elucidate the site of inhibition in the adrenal gland. Although ketoconazole impaired adrenocorticotropic hormone-stimulated cyclic (c)AMP production, dibutyryl cAMP addition did not bypass the steroidogenic blockade indicating that the critical ketoconazole-inhibited step was distal to cAMP. Addition of radiolabeled substrates to isolated adrenal cells and analysis of products by high performance liquid chromatography demonstrated a ketoconazole block between deoxycorticosterone (DOC) and corticosterone. This 11-hydroxylase step is carried out by a P450-dependent mitochondrial enzyme. No restriction of progesterone or pregnenolone conversion to DOC was detected, steps carried out by non-P450-dependent microsomal enzymes. Inhibition of cholesterol conversion to pregnenolone by mitochondrial fractions indicated a second block at the side chain cleavage step, another mitochondrial P450-dependent enzyme. Adrenal malate dehydrogenase, a non-P450-dependent mitochondrial enzyme was not inhibited while renal 24-hydroxylase, a P450-dependent mitochondrial enzyme in another organ, was blocked by ketoconazole. We conclude that ketoconazole may be a general inhibitor of mitochondrial P450 enzymes. This finding suggests that patients receiving ketoconazole be monitored for side effects relevant to P450 enzyme inhibition. Further, we raise the possibility that this drug action may be beneficially exploited in situations where inhibition of steroidogenesis is a therapeutic goal.

INTRODUCTION

Ketoconazole has been an efficacious antimycotic agent because of its minimal toxicity and its effectiveness after oral administration against a wide range of fungal pathogens (1). However the appearance of gynecomastia in a few patients (2) was suggestive of a drug-hormone interaction. We and others recently showed that ketoconazole interfered with the production of adrenal (3) and gonadal steroids (4) both in patients and rat in vitro systems. These findings raised the possibility that ketoconazole, or a specifically designed analogue, could have additional utility as an inhibitor of steroidogenesis in the myriad clinical settings where reduction of steroid production would be useful, including Cushing's syndrome, hirsutism, and hormone-dependent cancers. We therefore pursued our studies of ketoconazole action to elucidate the step(s) in the steroidogenic pathway that were blocked by this antimycotic agent. The present paper demonstrates that ketoconazole inhibits adrenal mitochondrial P450-dependent enzymes. We detected inhibition of both the cholesterol side chain cleavage step as well as the 11-hydroxylase step. In addition, a kidney mitochondrial P450 enzyme, 25-hydroxy vitamin D-24-hydroxylase, was also blocked, suggesting that ketoconazole may have the general property of inhibiting P450-dependent enzymes. In the light of these findings additional side effects of ketoconazole relevant to P450 inhibition should be closely monitored. Moreover, we believe that studies to exploit this enzyme-blocking activity of ketoconazole in new clinical situations are now warranted.

METHODS

Adrenal preparations. Female Sprague-Dawley rats, 220-300 g, were decapitated and the adrenal glands re-
moved. Isolated adrenal cells were prepared by collagenase
digestion as previously described (3). DNA was measured by
the diphenylamine method (5). Rats that were ether-stressed
for 10 min to raise endogenous adrenocorticotropic hormone
(ACTH) were used to prepare adrenal mitochondria by the
method described by Mason et al. (6).

Corticosterone and cyclic (c)AMP. Total cAMP (cells and
medium combined) was extracted into 6% trichloroacetic
acid, neutralized by the addition of CaCO3 (7), and mea-
sured by radioimmunoassay (Becton, Dickinson & Co., Or-
angeburg, NY). Corticosterone was extracted from the me-
dium with methylene chloride and measured by radioim-
monoassay as previously described (3).

Steroidogenic studies. After 15 min of pretreatment of
isolated cells or mitochondria with ketoconazole or vehicle
(saline), radioactive substrates were added (0.5-1.0 μCi/ml)
to begin the assay. Incubations were carried out for 5-120
min at 37°C under 95% O2:5% CO2. The reactions were
stopped by centrifugation and radiolabeled products in the
medium were extracted in 10 vol of chloroform/methanol,
2:1 (8). Following a wash with methanol/saline/chloroform
47:50:3 (Folch wash), the organic phase was taken to dryness
under nitrogen. Samples were redissolved in 70% methanol
and an aliquot was injected into a Varian model 5000 high
performance liquid chromatograph (HPLC) equipped with
a 30 cm reverse-phase column (Varian MCH-10, Varian
Associates, Inc., Palo Alto, CA). The mobile phase was
methanol and water. [3H]Cholesterol (47 Ci/mmol),
[3H]deoxy corticosterone ([3H]DOC) (40 Ci/mmol), [3H]-
progesterone (51 Ci/mmol), and [14C]pregnenolone (56 mCi/
mol) were all purchased from Amersham Corp. (Arlington
Heights, IL). Radioinert steroids used for standards were
purchased from Steraloids, Inc., (Wilton, NH).

24-Hydroxylase activity was assessed in a cultured pig
kidney cell line (LLC-PK1) by measuring the conversion of
25-hydroxy[3H]vitamin D3 (Amersham Corp., 20 Ci/mmol)
to 24,25(OH)2D3 by an HPLC method (9). Adrenal mito-
chondrial malate dehydrogenase activity was measured by
the method of Hoppel and Cooper (10).

RESULTS AND DISCUSSION

The first experiments were designed to evaluate the possi-
bility that ketoconazole interfered with the initial steps of
ACTH-stimulated corticosterone production. We reason-
ed that measurement of cAMP generation would allow
detection of an inhibitor action at the level of the ACTH
receptor, the regulatory protein or the adenylate cyclase
system. As shown in Table I, ketoconazole did partially inhibit cAMP generation. However, the reduction in cAMP
was disproportionately less than the ketoconazole effect to inhibit corticosterone production (3) and therefore did not seem
adequate to explain the steroidogenic block. This is es-
pecially true in the light of the excess cAMP that is
known to be generated in relation to that required for
steroidogenesis (11).

<table>
<thead>
<tr>
<th>Condition</th>
<th>cAMP pmol/μg DNA</th>
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<tbody>
<tr>
<td>Basal</td>
<td>1.2±0.2</td>
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<tr>
<td>Ketoconazole</td>
<td>2.6±0.8</td>
</tr>
<tr>
<td>ACTH</td>
<td>6.2±1.8</td>
</tr>
<tr>
<td>ACTH + ketoconazole</td>
<td>3.1±0.4</td>
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Total cAMP (cells plus medium) was measured by radioim-
monoassay after 30 min of incubation in the presence of ACTH (5
ng/ml) and/or ketoconazole (5 μg/ml). Values shown are means±SE
of five experiments.

To ascertain whether the cAMP inhibition played a role in the ketoconazole blockade of corticosterone production, we performed a dibutyryl cAMP (bt2cAMP)
"bypass" experiment. As shown in Table II, ketocon-
azole inhibited corticosterone production despite bt2cAMP addition. The inability of bt2cAMP to bypass the
ketoconazole blockade indicated that the impair-
ment in steroidogenesis was distal to the cAMP
production step.

We next sequentially evaluated the three major dis-
tal steroidogenic enzyme steps by making additions of
the relevant radiolabeled substrates to isolated adrenal
cells and analyzing the products by HPLC (Fig. 1). With
[3H]DOC as substrate, ketoconazole inhibition of 11-hydroxylase activity was observed. Using [3H]-
progesterone as substrate, conversion to DOC was
demonstrated showing substantial 21-hydroxylase ac-
tivity; accumulation of DOC confirmed the 11-hy-
droxylase block shown previously. Similarly, with [14C]-
pregnenolone as substrate, conversion to progesterone
and DOC but not corticosterone was detected demo-
strating the presence of 3-β-hydroxysteroid dehy-

<table>
<thead>
<tr>
<th>Condition</th>
<th>Corticosterone ng/μg DNA</th>
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<tbody>
<tr>
<td>Basal</td>
<td>50±14</td>
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<tr>
<td>Ketoconazole</td>
<td>23±9</td>
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<tr>
<td>bt2cAMP</td>
<td>136±5</td>
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<tr>
<td>bt2cAMP + ketoconazole</td>
<td>26±11</td>
</tr>
</tbody>
</table>

Corticosterone production was measured by radioimmuno-
assay after 60 min of incubation in the presence of bt2cAMP (1.0 mM)
and/or ketoconazole (5 μg/ml). Values shown are means±SE for
four experiments.

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1 Abbreviations used in this paper: ACTH, adrenocorticotrophic
hormone; bt2cAMP, dibutyryl cAMP; cAMP, cyclic
AMP; DOC, deoxycorticosterone; HPLC, high performance
liquid chromatograph(y).

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Ketoconazole, Δ⁵-3 oxosteroid isomerase, and 21-hydroxylase activities and again confirming blockade of 11-hydroxylase activity. In experiments not shown, these effects of ketoconazole were demonstrated to be dose dependent between 0.1 µg/ml (minimal blockade) and 5 µg/ml (almost complete blockade).

To test the cholesterol side-chain cleavage reaction, [³H]cholesterol was added as substrate to an adrenal mitochondrial preparation. As shown in Fig. 2, cholesterol conversion to pregnenolone and more polar metabolites was well demonstrated in control samples but was significantly impaired in ketoconazole-treated samples. Similar experiments in mitochondrial preparations with [³H]DOC as substrate demonstrated impairment of 11-hydroxylase activity but to a lesser degree than side-chain cleavage activity (data not shown).

Since the two blocked enzyme systems (side-chain cleavage and 11-hydroxylase) are both P450 dependent and mitochondrial in location, we thought it of interest to evaluate ketoconazole action on another mitochondrial P450-dependent enzyme in a different organ and on a non-P450-dependent adrenal mitochondrial enzyme. As can be seen in Fig. 3, ketoconazole caused a dose-dependent inhibition of renal 24-hydroxylase, a P450-dependent enzyme, in LLC-PK₁ cells. Of note is that the concentration of ketoconazole achieving 50% inhibition in the renal cells (0.2 µg/ml) is in close agreement with the concentration inhibiting 50% corticosterone production in adrenal cells, 0.3 µg/ml (3). In data not shown, mitochondrial adrenal malate dehydrogenase activity, a non-P450-dependent enzyme, was not inhibited by concentrations of ketoconazole as high as 10 µg/ml.

In summary, the data presented in this paper indicate that three mitochondrial P450-dependent enzymes in two different organs were inhibited by ketoconazole. No inhibition of non-P450-dependent enzymes was detected in three adrenal microsomal enzymes (3-β-hydroxysteroid dehydrogenase, Δ⁵-3 oxosteroid isomerase and 21-hydroxylase) nor one adrenal mitochondrial enzyme (malate dehydrogenase). We hypothesize that ketoconazole may be a general inhibitor of mitochondrial P450 enzymes. Since peak levels of ketoconazole in patients taking 200 or 400 mg of drug per day are between 2 and 20 µg/ml (12), therapeutically achieved concentrations of drug are effective in inhibiting these enzymes and thus steroidogenesis. This finding is in accord with the previous in vivo studies (3, 4).

- 70% increasing linearly to 100% between 15 and 17 min, and continuing at 100% for the remaining elution period. The standards were run in the same system: B, corticosterone; DOC, deoxycorticosterone; Prog, progesterone; Preg, pregnenolone.

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The postulated mechanism of action of ketoconazole, inhibition of fungal sterol synthesis, is presumed to occur at the 14-demethylation step in the conversion of lanosterol to ergosterol (13). Since this mixed function oxidase may also be P450 dependent, it perhaps should not be surprising that similar mammalian enzymes are also affected. In the light of the current findings, what is most striking is the lack of toxicity...
of ketoconazole noted thus far. This may relate to the pharmacokinetics of ketoconazole and the requirement for near peak concentrations of drug to inhibit the enzymes.

The information developed here regarding the actions of ketoconazole on mammalian P450-dependent enzymes indicates the need for continued careful monitoring of patients receiving this drug to detect specific side effects involving these very important enzyme systems. This is especially pertinent if the dose and/or frequency of ketoconazole administration are increased. On the other hand, we believe that this action of ketoconazole, to impair steroid hormone synthesizing activity, may be usefully exploited in those clinical settings where inhibition of steroidogenesis would be a beneficial therapeutic goal.

ACKNOWLEDGMENT

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REFERENCES