J. steroid Biochem. Vol. 23, No. 6A, pp. 991–994, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

EFFECT OF KETOCONAZOLE AND MICONAZOLE ON 25-HYDROXYVITAMIN D_3 METABOLISM BY CULTURED CHICK KIDNEY CELLS*

HELEN L. HENRY
Department of Biochemistry, University of California, Riverside, CA 92521, U.S.A.

(*Received* 22 *May* 1985)

Summary—The antifungal imidazoles, ketoconazole and miconazole, were tested for effects on 25-hydroxyvitamin D_3 metabolism in primary cultures of chick kidney cells. Both behave as competitive inhibitors of 1-hydroxylation of 25-OH-D_3 with approximate K_i's of 0.8 and 5.0 μM for ketoconazole and miconazole, respectively. Ketoconazole was as effective when added at the same time as the substrate as when the cells were preincubated with the compound. Ketoconazole also inhibited the production of 24,25$(OH)_2D_3$ in cells in which this activity was induced by 1,24$(OH)_2D_3$. The data suggest that therapeutic doses of these antifungal imidazoles could affect vitamin D status and calcium metabolism.

INTRODUCTION

Ketoconazole and miconazole are broad spectrum, antifungal agents. They are believed to exert their effects through interference with ergosterol synthesis and the resulting defective membrane biosynthesis [1, 2]. A small proportion of males treated with ketoconazole have been reported to develop gynecomastia [3, 4], a finding which led to the investigation of the effect of this compound on gonadal and, subsequently, adrenal steroid synthesis. Serum testosterone levels were decreased in normal individuals given therapeutic doses of ketoconazole and testosterone synthesis by isolated rat Leydig cells was inhibited by ketoconazole added *in vitro* [6]. Similarly, serum levels of cortisol in response to ACTH in normal individuals were dramatically, although transiently, reduced by ketoconazole treatment and this compound abolished corticosterone synthesis by isolated rat adrenal [6] and mouse adrenal tumor [7] cells. Evidence has been presented that the enzymes blocked by ketoconazole were the cholesterol side chain cleavage system and the 11 β-hydroxylase [7, 8]. Ketoconazole inhibited liver microsomal cytochrome P-450 mediated drug metabolism [9] and it, along with miconazole and clotrimazole, are potent inhibitors of human placental aromatase acitivity [10]. In addition, it has been reported that the 24-hydroxylation of 25-hydroxyvitamin D_3 by a kidney cell line was also inhibited by ketoconazole [8].

25-Hydroxyvitamin D_3 (25-OH-D_3) is produced in the liver from vitamin D_3 and is the major circulating form of vitamin D_3. It is further hydroxylated in the kidney, primarily to one of the two dihydroxylated metabolites, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) or 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$). In terms of stimulating intestinal calcium transport and bone mineral mobilization, 1,25$(OH)_2D_3$ is by far the most potent of the vitamin D metabolites, while the biological role of 24,25$(OH)_2D_3$ is as yet not entirely clear (see reference 11 for review). The present study was undertaken to extend the observation [8] regarding the impact of azole antifungal agents on the metabolism of 25-hydroxyvitamin D_3.

EXPERIMENTAL

Primary cultures of chick kidney cells were prepared essentially as described previously [12]. The kidneys of 14–18 day old chicks, fed a vitamin D-deficient diet since hatching, were perfused, removed, and minced. Fragments were incubated with collagenase (Sigma Type I, 0.6 mg/ml) and hyaluronidase (Sigma Type I, 0.5 mg/ml) for 10 min and 0.12% trypsin (GIBCO) for 1 min. Following a series of centrifugations to obtain a suspension consisting primarily of single cells, the cells were diluted to 5×10^5 cells per ml in Minimal Essential Medium with Earle's salts (MEM; GIBCO) containing 10% fetal calf serum (Microbiological Associates), and antibiotics (penicillin and streptomycin, 100 μml). The following day, old medium and unattached cells were discarded, and fresh MEM containing 5% fetal calf serum was added. Cultures were routinely used 96 h after plating and 20–24 h after being changed to serum-free medium containing 5 μg/ml insulin. At this time, the cultures were approx 90% confluent.

To assay 25-OH-D_3 metabolism, 25-hydroxy-[26,27-^{3}H] vitamin D_3 (Amersham, 22 Ci/mmol) was added in 5 μl ethanol to a final concentration of 5×10^{-8} M, unless indicated otherwise. Also, unless indicated otherwise, ketoconazole was present for 1 h prior to the addition of the radioactive substrate. The routine time of incubation was 30 min, at which time

*Supported by USPHS Grant AM-21398.

the cells and medium were collected in methanol–chloroform, 2:1 (v:v). Lipids were extracted by a modification of the procedure of Bligh and Dyer[13]. The chloroform was evaporated under a stream of nitrogen and the dried lipids redissolved in hexane and filtered. Following evaporation of the hexane under nitrogen, the samples were taken up in 100 μl of hexane and the radioactive metabolites separated by high pressure liquid chromatography. This was carried out with a Varian model 5000 chromatograph equipped with a Waters Radial Compression Module containing a 10 μm silica cartridge. A 3–15% gradient of isopropanol in hexane was applied and the u.v. absorption of added authentic steroids was used to establish retention times of the eluted radioactive metabolites. Fractions were collected and the radioactivity associated with the substrate, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ peaks determined by liquid scintillation counting.

Ketoconazole and miconazole (Janssen Pharmaceuticals) were the generous gift of Dr Larry E. Vickery.

Data are presented as the mean of quadruplicate samples ± the standard deviation.

RESULTS AND DISCUSSION

In the experiment depicted in Fig. 1, one set of cultures was treated with 10^{-7} M 1,25$(OH)_2D_3$ for the 20 h period in serum-free medium prior to assay. This treatment depresses 1-hydroxylase and induces 24-hydroxylase activity [12]. The effect of various concentrations of ketoconazole on both enzyme activities in these cultures as well as in cultures not pretreated with 1,25$(OH)_2D_3$ was then assessed. At the highest concentration used, ketoconazole brought about a 70% (approximately) decrease in both enzyme activities. The results were the same for the 1-hydroxylase regardless of whether the cultures had been pretreated with 1,25$(OH)_2D_3$. Pharmacokinetic studies in human subjects have shown that these μg/ml concentrations of ketoconazole are obtained in the serum within a few hours following an oral therapeutic dose of 200–600 mg [13].

As shown in Fig. 2 the rate of production of [^{3}H]1,25$(OH)_2D_3$ was linear for the 30 min incubation with radioactive substrate both in the presence and absence of ketoconazole. This suggests that it is indeed the 1-hydroxylase that is being interfered with and not, for example, accelerated destruction of the product.

The results of the experiment shown in Fig. 3 indicate that the effect of ketoconazole was immediate, with maximal inhibition achieved when it was added at the same time as the substrate. One hypothesis which was originally put forth [5] to explain the effect of ketoconazole on the production of glucocorticoids and androgens was that it interfered with the availability of cholesterol as a substrate for steroidogenesis. This is unlikely, given the fact that it also inhibits 11-β-hydroxylation of deoxycorticosterone [8] as well as the 1- and 24-hydroxylation of 25-OH-D_3 and that it acts quite rapidly as shown in Fig. 3.

Interestingly, there is a modest but statistically significant "escape" from inhibition at 1 and 6 h which is reminiscent of the "escape" in terms of serum testosterone [5] and cortisol [6] levels observed in humans. In the latter cases, the return of steroid concentrations to baseline levels was attributed to clearance of the drug from the circulatory system. It is possible that these kidney cell cultures are indeed

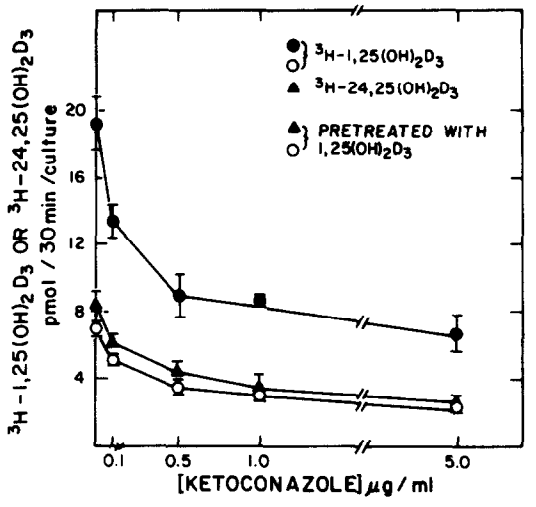

Fig. 1. Effect of ketoconazole on [^{3}H]25-OH-D_3 metabolism in chick kidney cell cultures. Cultures were in serum-free medium for 20 h prior to assay and ketoconazole (5 μg/ml) was added 1 h before the addition of substrate. To one set of cultures (△, ○) 10^{-7} M 1,25$(OH)_2D_3$ was added when cells were changed to serum-free medium. The assay of [^{3}H]25-OH-D_3 metabolism was carried out as described in Experimental. Values are the mean of 4 cultures ± SD.

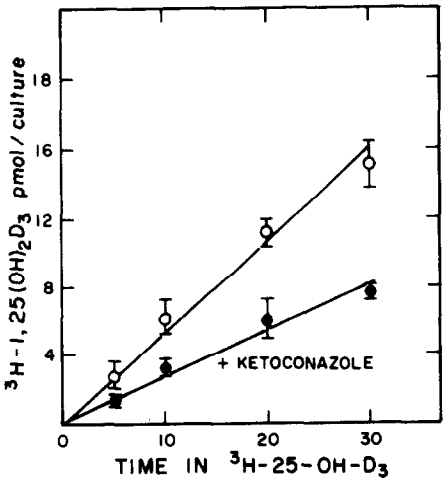

Fig. 2. Time course of [^{3}H]1,25-$(OH)_2D_3$ production by chick kidney cell cultures in the presence and absence of ketoconazole. Ketoconazole (5 μg/ml) was added 1 h prior to the addition of radioactive substrate to initiate the reaction. Values are the mean of 4 cultures ± SD.

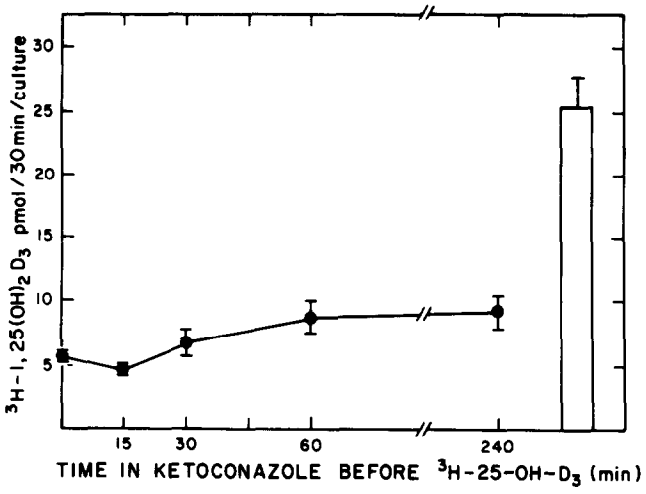

Fig. 3. Time required for ketoconazole to block [^{3}H]1,25-$(OH)_2D_3$ production by chick kidney cell cultures. Ketoconazole (5 μg/ml) was added to the cultures the indicated time before the addition of radioactive substrate to initiate the reaction. The open bar represents cultures to which no ketoconazole was added. Values are the mean of 4 cultures $\pm$ SD.

metabolizing ketoconazole to an inactive form. A comparison of the clearance rate of the drug in patients with renal failure and one with hepatic insufficiency suggested that the liver, rather than the kidney, is the principle site of removal of ketoconazole from the circulation [14], but a role for the kidney in the metabolism of these drugs has not been ruled out.

Additional experiments were carried out in which the substrate concentration was varied at several different ketoconazole and miconazole concentrations. Typical double reciprocal plots of the data are shown in Fig. 4. Approximate K_i's of 5.0 μM (2.4 μg/ml) and 0.8 μM (0.4 μg/ml) were obtained in this whole cell system for miconazole and ketoconazole, respectively. Both behaved as competitive inhibitors of 1-hydroxylation. Clearly, ketoconazole is by far the more effective of the two inhibitors. The half-maximal substrate concentration in these cells is approx 0.1 μM, so both inhibitors apparently bind less tightly to the 1-hydroxylase than does the substrate. This is in contrast to the situation with the placental microsomal aromatase for which the K_i for miconazole was 0.06 M and the K_m for the substrate, androstenedione, was 0.22 μM [10]. Interestingly, miconazole was the more potent inhibitor of the aromatase (by a factor of 2) whereas ketoconazole was (by 3-fold) more potent than miconazole in inhibiting cholesterol side-chain cleavage [10].

In summary, the data presented here indicate that the inhibitory effects of ketoconazole extend beyond the steroidogenic pathways involved in glucocorticoid, androgen and estrogen biosynthesis and that it, as well as miconazole, could have an impact on vitamin D metabolism as well. It would be of great interest to determine the effect of therapeutic doses of the imidazole antifungal agents on serum levels of 25-OH-D_3 (produced by a cytochrome P_{450}-dependent mitochondrial enzyme in the human liver [15]. 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ as well as other parameters of calcium metabolism. It has been

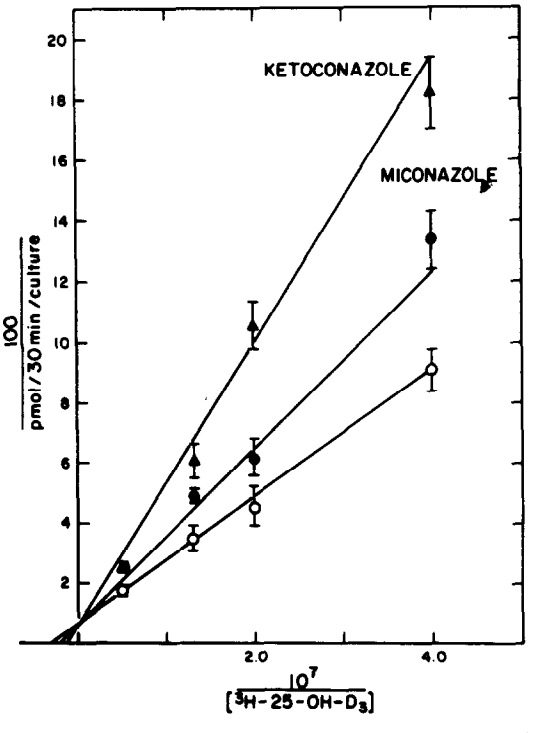

Fig. 4. Double reciprocal plot of kinetic data for 1,25$(OH)_2D_3$ production. The substrate concentration was varied in the absence and presence of 0.75 μg/ml of ketoconazole or miconazole, both of which were added 1 h prior to the addition of radioactive sustrate. Values are the mean of 4 determinations per point, $\pm$SD.

suggested that it is likely that increased daily doses of ketoconazole and miconazole will be used clinically in an attempt to combat more resistant fungal infections, in which case patients should be monitored for possible side effects due to deranged glucocorticoid (and presumably mineralocorticoid) and androgen production [5, 6]. Clearly, the same can be said for the vitamin D-derived steroids involved in calcium metabolism.

Acknowledgements—The author is grateful to Larry E. Vickery for helpful discussions of these experiments and to Eden M. Luntao for her expert technical contributions.

REFERENCES

1. Van den Bossche H., Willemsens G., Cools W., Cornelissen F., Lauwers W. F. and Van Sutsem J. M.: *In vitro* and *in vivo* effects of the antimycotic drug ketoconazole on sterol synthesis. *Antimicrob. Agents Chemother.* **17** (1980) 922–928.
2. Willemsens G., Cools W. and Van den Bossche H.: Effects of miconazole and ketoconazole on sterol synthesis in a subcellular fraction of yeast and mammalian cells. In *The Host Invader Interplay* (Edited by H. Van den Bossche). Elsevier North Holland, New York (1980) pp. 691–694.
3. DeFelice R., Johnson D. G. and Galgini J. N.: Gynecomastia with ketoconazole. *Antimicrob. Agent Chemotherap.* **19** (1981) 1073–1074.
4. Stevens D. A., Stiller R. L., Williams P. L. and Sugar A. M.: Experience with ketoconazole in three major presentations of progressive coccidiodomycosis. *Am. J. Med.* **74** (1984) 58–63.
5. Pont A., Williams P. L., Azhar S., Retz R. E., Bochra C., Smith E. R. and Stevens D. A.: Ketoconazole blocks testosterone synthesis. *Archs int. Med.* **142** (1982) 2137–2140.
6. Pont A., Williams P. A., Loose D. S., Feldman D., Reitz E., Bochra C. and Stevens D. A.: Ketoconazole blocks adrenal steroid synthesis. *Archs int. Med.* **97** (1982) 370–372.
7. Kowal J.: The effect of ketoconazole on steroidogenesis in cultured mouse adrenal cortex tumor cells. *Endocrinology* **112** (1983) 1541–1543.
8. Loose D. S., Kan P. B., Hirst M. A., Marcus R. A. and Feldman D.: Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. *J. clin. Invest.* **71** (1983) 1495–1499.
9. Sheets J. J. and Mason J. I.: Ketoconazole: A potent inhibitor of cytochrome P-450 drug metabolism in rat liver. *Drug metab. Dist.* **12** (1984) 603–606.
10. Mason J. I., Murry B. A., Olcott M. and Sheets J. J.: Imidazole antimycotics: Inhibitors of steroid aromatase. *Biochem. Pharmac.* **34** (1985) 1087–1092.
11. Henry H. L. and Norman A. W.: Vitamin D: Metabolism and biological actions. *A. Rev. Nutr.* **4** (1984) 493–520.
12. Henry H.: Regulation of the hydroxylation of 25-hydroxyvitamin D_3 *in vivo* and in primary cultures of chick kidney cells. *J. biol. Chem.* **254** (1979) 2722–2729.
13. Bligh E. G. and Dyer W. J.: A rapid method of total lipid extraction and purification. *Can. J. biochem. Physiol.* **37** (1959) 911–917.
14. Brass C., Galgini J. N., Blaschke T. F., DeFelice R., O'Reilly R. A. and Stevens D. A.: Disposition of ketoconazole, an oral antifungal, in humans. *Antimicrob. Agents Chemother.* **21** (1982) 151–158.
15. Saaren K., Bergseth S., Oftebro H. and Pederse J.: Subcellular localization of vitamin D_3 25-hydroxylase in human liver. *J. biol. Chem.* **259** (1984) 10936–10940.