Ketoconazole Blocks Bile Acid Synthesis in Hepatocyte Monolayer Cultures and In Vivo in Rat by Inhibiting Cholesterol 7α-Hydroxylase

Hans M. G. Princen, Christine M. G. Huijsmans, Folkert Kuipers,* Roel J. Vonk,* and Herman Jan M. Kempen
*TNO Gaubius Institute for Cardiovascular Research, 2313 AD Leiden; *Department of Pediatrics,*
University of Groningen, 9712 KZ Groningen, The Netherlands

Abstract

In cultured hepatocytes conversion of [4-^{14}C]cholesterol into bile acids was dose dependently reduced by the antimycotic drug ketoconazole, giving half-maximal inhibition at 10 μM ketoconazole in rat hepatocytes and at 1 μM in human hepatocytes. No change was observed in the ratio of produced cholic, β-muricholic, and chenodeoxycholic acid with increasing amounts of the drug. Conversion of [4-^{14}C]7α-hydroxycholesterol, an intermediate of bile acid pathway, to bile acids was not affected by ketoconazole. These results together with kinetic studies with rat liver microsomes, demonstrating noncompetitive inhibition (K_i = 0.4 μM), indicate that cholesterol 7α-hydroxylase is the main site of inhibition.

In bile-diverted rats a single dose of ketoconazole (50 mg/kg) dramatically impaired bile flow and biliary bile acid output (92% inhibition). A similar blockade was observed using [4-^{14}C]cholesterol as precursor for bile acid synthesis. Therefore, treatment of patients with this drug may inhibit bile acid synthesis, resulting in a reduction of the bile acid pool size after long-term ketoconazole therapy.

Introduction

Ketoconazole is an orally active imidazole derivative and antimycotic agent with minimal toxicity, effective against a wide range of fungal pathogens (1). Its principal action is based on inhibition of the cytochrome P-450–mediated 14α-demethylation of lanosterol in ergosterol synthesis in fungi at low concentrations. Much higher concentrations of the drug are required to inhibit conversion of lanosterol to cholesterol in man (2), rat (3), and in cultured lymphocytes (4) and Hep G2 cells (5). However, the last few years several side effects have been reported. Long-term ketoconazole therapy increased serum triglyceride levels in humans (6). Gynecomastia was observed in a few patients (7), as a result of interference of the drug with testosterone synthesis by inhibition of the C$_{17-20}$ lyase (8, 9). Moreover, ketoconazole blocked adrenal steroidogenesis (10, 11) by inhibiting adrenal mitochondrial P450–dependent enzymes (11-hydroxylase of deoxycorticosterone and side chain cleavage of cholesterol) (11). In addition, these investigators found that a kidney

mitochondrial P-450 enzyme was blocked and they suggested that the drug may be a general inhibitor of mitochondrial P-450 enzymes (11). Recently, however, ketoconazole has also been shown to be a potent inhibitor of drug N-demethylase and O-deethylase activity in liver microsomes from rats (12, 13). Similar effects were observed on hepatic oxidative drug metabolism in humans (14).

In the light of the effects of ketoconazole on sterol synthesis, we studied the effect of this compound on bile acid synthesis, in which several cytochrome P-450–dependent enzymes are involved (15). This study demonstrates that ketoconazole inhibits bile acid synthesis in cultured human and rat hepatocytes. The reduced synthesis was the result of potent inhibition of the rate-limiting enzyme in the pathway of bile acid synthesis, cholesterol 7α-hydroxylase (16). This enzyme, localized in microsomes, also belongs to the class of cytochrome P-450 enzymes (16).

In in vivo experiments, performed in rats with chronic total bile diversion (17, 18), ketoconazole administration caused a dramatic effect on bile flow and biliary bile acid output.

Methods

Materials and animals. Ketoconazole was obtained from Janssen Life Sciences Products, Beerse, Belgium. [4-^{14}C]Cholesterol (60 mCi/mmol), [^{3}H(G)]taurocholic acid (6.6 Ci/mmol) and [24-^{14}C]taurocholic acid (59 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Williams E culture medium and fetal bovine serum were from Flow Laboratories, Irvine, Scotland. Collagenase type I, soybean trypsin inhibitor, bovine serum albumin, choloylglycine hydrolase, pyruvic acid, dithiothreitol (DTT),[1] butylated hydroxytoluene (BHT), Tween 80, and EGTA were obtained from Sigma Chemicals (St. Louis, MO). Cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), chenodeoxycholic acid (3α,7α,dihydroxy-5β-cholanoic acid) and 7α- and 7β-hydroxycholesterol were from Steraloids (Wilton, NH). Glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase, NADH and lactate dehydrogenase (from rabbit muscle) were purchased from Boehringer (Mannheim, FRG). Insulin (Actrapid, 40 IU/ml) was from Novo Industri (Copenhagen, Denmark) and dexamethasone from Organon (Oradexon, 5 mg/ml, Oss, The Netherlands). Trypan blue was from BDH Chemicals (Poole, Dorset, England). Cholesterol, Hepes, thin-layer chromatography (TLC)–precoated silica gel 60 plates and all salts and solvents were obtained from E. Merck (Darmstadt, FRG). β-Muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid) was prepared from bile of bile-diverted rats after extraction (19) and deconjugation with potassium hydroxide, as described (20).

[4-^{14}C]7α-Hydroxycholesterol was biosynthesized in the cholesterol 7α-hydroxylase assay by performing eight parallel incubations for 90 min with microsomes (1.5 mg protein) from rats kept on a reversed light-dark cycle for 3 wk and fed 4% cholestyramine during the last week. Cholesterol 7α-hydroxylase activity of these microsomes was 10-fold

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1. *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; DTT, dithiothreitol; K$_i$, inhibitor constant; TLC, thin-layer chromatography.

control value; yield of [^{14}C]7α-hydroxycholesterol after extraction and TLC was 5.6% of added [4-^{14}C]cholesterol (10 μCi). In these experiments no unlabeled exogenous cholesterol was added to the incubation mixture.

Male Wistar rats 200–300 g (250–300 g for hepatocyte isolation) were used throughout and maintained on standard chow (Hope Farms, Woerden, The Netherlands) and water ad lib. For preparation of hepatocytes and microsomes animals were killed between 9 and 10 a.m.

Rat hepatocyte preparation and culture. Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously (21). Viability, as determined by trypan blue exclusion, was higher than 90%. The cells were seeded on 100-mm diameter plastic tissue culture dishes or 6-well cluster plates (35-mm diam, Costar, Cambridge, MA) at a density of 1 × 10^5 cells/cm^2 and were maintained in Williams E medium (22) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 20 mU/ml insulin, 50 nM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO$_2$/95% air atmosphere. After a 4-h attachment period medium was refreshed with 5 ml (dishes) or 1 ml (wells) of culture medium and [4-^{14}C]cholesterol or [4-^{14}C]7α-hydroxycholesterol was added, solubilized in fetal bovine serum. After a 24-h incubation period with 0 to 20 μM ketoconazole, viability was determined. Cells at all concentrations were viable, as judged by trypan blue exclusion (>95%) and leakage of the cytoplasmic enzyme lactate dehydrogenase to the culture medium. Lactate dehydrogenase activity (determined as described in ref. 23) in culture medium was 10% of cellular content, in agreement with others (24, 25), and not significantly different with addition of 20 μM ketoconazole.

Human hepatocytes. Human hepatocytes were isolated from a piece of liver tissue, essentially as described before (26). After informed consent of relatives was obtained, the liver was taken during routine human donor nephrectomy from a physically healthy 39-yr-old male who died after severe traumatic brain injury. Immediately after kidney removal, a 200-g segment of liver tissue was excised and immersed in a cold buffer containing 10 mM Hepes, pH 7.4, 142 mM NaCl, 6.7 mM KCl, and 0.5 mM EGTA. The liver was transported to the perfusion site within 30 min, and perfusion with 1 liter of this buffer at a rate of 40 ml/min was started after insertion of four polyethylene catheters (18 gauge) in vascular orifices that could be identified at the cut surface. After the preperfusion the liver was perfused successively with 100 ml of a Hepes buffer (pH 7.6) without recirculation, and 250 ml of this buffer containing 0.05% collagenase with recirculation for 20 min. Liver tissue was dissociated in Hanks' buffer containing 2% bovine serum albumin, cells were filtered through a 250-μm filter, centrifuged (75 g for 5 min), and washed three times in cold culture medium to remove damaged and nonparenchymal cells. After counting and testing for trypan blue exclusion (viability was 78%), hepatocytes were plated at a density of 1.25 × 10^5 cells/cm^2 and cultured as described for rat hepatocytes. The medium was renewed after 6 h and every 24 h thereafter.

Bile acid synthesis in cultured hepatocytes. After 7, 17, or 24 h incubation with [4-^{14}C]cholesterol cells and media from rat hepatocyte cultures were harvested and bile acids were extracted using a reverse-phase cartridge (Sep-pak C-18, Waters Associates, Milford, MA) with [^{3}H(G)]taurocholic acid as recovery standard essentially as in (27). Bile acids were deconjugated using choloylglycine hydrolase and after extraction separated on a thin layer of silica. The plates were developed in toluene/dioxane/glacial acetic acid 20:10:2; vertical edges were colored in iodine vapor to visualize reference bile acids or plates were autoradiographed after spraying with Enhance (New England Nuclear, Boston, MA). Areas containing bile acids were scraped off and counted using a double-label program. Bile acid markers used with their retardation factor (R_F)-values were cholic acid (0.22), β-muricholic acid (0.30), and chenodeoxycholic acid (0.48). Recovery of uniformly labeled [^{3}H(G)]taurocholate, after correction of 14% loss of radioactivity by deconjugation, was 75–95%, depending on the amount of protein in the sample. In medium and cells from human hepatocyte cultures (cultured from 42 to 66 h after plating) radioactivity was determined in cholic and chenodeoxycholic acid, known to be the major primary bile acids in man (28, 29).

Preparation of microsomes. Animals were anesthetized with Nembutal (Ceva, Paris, France). Livers were perfused in situ and homogenized in an ice-cold buffer containing 100 mM sucrose, 40 mM potassium phosphate, pH 7.4, 50 mM potassium fluoride, 2 mM EDTA, and 5 mM DTT. All subsequent procedures were performed at 4°C. After centrifugation at 16,000 g for 20 min, microsomes were isolated by centrifugation of the supernatant for 1 h at 100,000 g. Microsomes were washed once in the same buffer without potassium fluoride and with 100 mM potassium phosphate, pH 7.4, resuspended in this last buffer at 15–25 mg/ml, frozen in liquid nitrogen in small portions, and stored at −80°C. Microsomal protein and cholesterol were determined according to Lowry et al. (30) and Gamble et al. (31), respectively.

Cholesterol 7α-hydroxylase assay. Cholesterol 7α-hydroxylase was measured as previously described (32) with minor modifications. Microsomes (0.5 mg protein) in duplicates were preincubated for 10 min at 37°C in a medium containing 50 mM potassium phosphate, pH 7.4, 2 mM DTT, 100 μM [4-^{14}C]cholesterol (0.5 μCi, purified before use) and 1.5 mg/ml Tween 80. Enzyme activity at the end of the preincubation time was assayed by addition of a NADPH regenerating system containing 20 mM glucose 6-phosphate, 2 mM NADP, 4 mM MgCl$_2$, and 1.4 IU glucose-6-phosphate dehydrogenase to a final volume of 1.0 ml. After 30 min at 37°C in a shaking water bath the reaction was stopped by addition of 10 ml chloroform/methanol 2:1 containing 0.01% BHT. The organic layer was washed with 2 ml 0.9% NaCl and evaporated. The residue, together with 7α- and 7β-hydroxycholesterol and cholesterol as markers, was applied to a thin layer of silica, which was twice developed with benzene/ethylacetate 2:3. 7α-Hydroxycholesterol and cholesterol, were visualized with rhodamine 6B or autoradiography, scraped off, and counted. Blank values, determined by running parallel incubations without a NADPH generating system, were subtracted before calculating enzyme activity. Formation of 7α-hydroxycholesterol was linear for at least 90 min and up to 1.5 mg added microsomal protein. Unless otherwise indicated ketoconazole (20 μl) was added dissolved in 50 mM HCl-1.5 mg/ml Tween 80.

In vivo experiments. Male Wistar rats (275–300 g) were housed individually in plexiglass cages in a temperature- and light-controlled room (temperature 20°C; lights on between 7 a.m. and 7 p.m.).

Rats were equipped with permanent silicon catheters in the bile duct, the duodenum, and the heart as described earlier (18). These catheters were tunneled subcutaneously and fixed to the skull, providing the opportunity either to return bile back to the animal via the duodenal catheter (closed enterohepatic circulation), or to drain the bile outside the cage (bile diversion). During bile diversion, bile was led through a polyethylene tubing with a swivel joint, thus permitting free movement to the rats, and allowing bile excretion to be studied in conscious and unrestrained animals. The heart catheter allowed intracardial administration as well as repeated blood sampling without additional stress to the animals (33). Experiments were performed after 8 d of biliary drainage, i.e., after establishment of a new steady state in bile acid synthesis (18). At 12 a.m. on the day of the experiment a single dose (50 mg/kg body wt) of ketoconazole in 50 mM HCl or an equivalent amount of solvent was injected via the duodenal catheter. During these experiments bile was collected continuously for 26 h after ketoconazole administration in tared test tubes in 2-h fractions, using a fraction collector. Before the experiment two fractions were collected to assess baseline rates of bile flow and biliary bile acid and cholesterol output. No difference was observed in food intake and body weight between ketoconazole-treated and control animals during the experiments.

In some experiments rat serum labeled with [4-^{14}C]cholesterol was injected intracardially 2 h after ketoconazole administration. For this purpose serum from a donor rat was incubated with [^{14}C]cholesterol (34), leading predominantly to association of radioactivity with the two high density lipoprotein subfractions in rat serum. All rats received 0.75 ml of serum containing 0.5 μCi [4-^{14}C]cholesterol as a bolus injection, whereas blood samples of 0.15 ml were taken at 5, 10, 15, and 30 min and 1, 2, 3, 6, 12, and 24 h after injection. Blood was immediately transferred to heparinized tubes and centrifuged (10 min, 3,500 g). Bile and plasma samples were stored at −20°C until analysis.

In another set of experiments ketoconazole was given to rats via the

duodenal catheter 3 h before intracardial administration of 0.25 μCi [24-^{14}C]taurocholic acid in saline. Bile was collected during 1.5 h in 15-min fractions.

Chemical analysis and calculations. Biliary bile acid concentration was measured using an enzymatic fluorimetric assay (Sterognost-Flu, Nyegaard, Oslo, Norway). Cholesterol concentrations in bile were determined after lipid extraction (19) according to Gamble et al. (31). Radioactivity in bile, after decolorization with an equal volume of H_2O_2, and plasma was measured in 50-μl aliquots by scintillation counting using an external standardization method. To distinguish between radiolabeled cholesterol and bile acids in bile, 50-μl portions were extracted (19), and radioactivity in the chloroform (cholesterol) and methanol-water phase (bile acids) was determined.

Biliary excretion rates of bile acids and cholesterol were calculated by multiplying bile flow by biliary concentration, the latter corrected for the dead space of the bile catheter. Excretion rates after the drug or acid injection (averaged per 2 h) were expressed in percentage of the baseline value in the last 4 h before administration. Statistical significance of differences was calculated using Student's t test for unpaired data (35) with the level of significance selected to be $P < 0.05$. Values were expressed as mean±SD.

Results

Synthesis of bile acids by cultures of rat hepatocyte monolayers. To avoid the influence of inhibition of cholesterol synthesis (by blocking 14α-demethylation of lanosterol) on bile acid synthesis from de novo synthesized cholesterol, we used [^{14}C]cholesterol as substrate for measuring bile acid synthesis. Fig. 1 shows the time course of bile acid synthesis over the 24-h period (4 to 28 h after plating), in which we studied the effect of ketoconazole on synthesis of bile acids by rat hepatocytes in monolayer culture. Cholic acid comprised 55% of total bile acid synthesis (considered to be the sum of all separately determined bile acids in medium and in cells) after 24 h of incubation, whereas chenodeoxycholic acid was only ~10% during the incubation period. At the three time points in this period 68–80% of total synthesized bile acids were found intracellularly. However, no specific retention of one of the three individual bile acids (cholic acid, β-muricholic acid, and chenodeoxycholic acid) in the cell was observed.

Effect of ketoconazole on bile acid synthesis by rat and human hepatocytes. To exclude the possibility that ketoconazole blocked

the uptake of cholesterol by the hepatocytes, we measured [4-^{14}C]cholesterol absorption by the cells during the 24-h incubation period with different concentrations of this drug. Approximately 3% of added [^{14}C]cholesterol per milligram cell protein was absorbed by the hepatocytes and no difference in [4-^{14}C]cholesterol uptake was found with 0, 5, and 20 μM ketoconazole. The total amount of radioactivity incorporated in bile acids in the absence of ketoconazole amounted to 3.5% of total [^{14}C]cholesterol present in the hepatocytes after 24 h of incubation. This last finding indicates that only a minor portion of added [4-^{14}C]cholesterol is converted to bile acids.

Addition of ketoconazole to monolayer cultures of rat hepatocytes caused a dose-dependent reduction of total bile acid synthesis by these cells (Fig. 2). No significant change was observed in the ratio of produced cholic acid (58±4% of total bile acid synthesis), β-muricholic acid (33±5%) and chenodeoxycholic acid (9±1%) with increasing amounts of ketoconazole. In contrast, there was a significant difference ($P < 0.05$) between bile acids associated with cells at 0, 1, 5, and 10 μM ketoconazole (75±3%, 76±7%, 76±7%, and 72±1% of total intracellular and extracellular bile acids, respectively) and at a concentration of 20 μM (60±5% of total bile acid synthesis). In these experiments no specific bile acid was retained by the hepatocytes.

A concentration as low as 5 μM ketoconazole inhibited bile acid synthesis in rat hepatocytes, whereas half-maximal inhibition (I_{50}) was achieved at 10 μM (Fig. 3). A similar blockade of bile acid synthesis by ketoconazole was observed with monolayer cultures of human hepatocytes. However, the I_{50}-value for bile acid synthesis by these human hepatocytes was approximately 10-fold lower than that found for rat hepatocytes.

Since cholesterol 7α-hydroxylase is the first enzyme in the bile acid synthesis pathway and believed to be the regulatory step (16), it was interesting to study the influence of ketoconazole on conversion of 7α-hydroxycholesterol into bile acids by rat hepatocyte monolayers. It has been shown previously that exogenous 7α-hydroxycholesterol is metabolized to bile acids (36, 37). The data presented in Table I demonstrate that >85% of the added [^{14}C]7α-hydroxycholesterol was converted into bile acids. Furthermore, in contrast with synthesis of bile acids from [4-^{14}C]cholesterol ketoconazole did not significantly inhibit bile
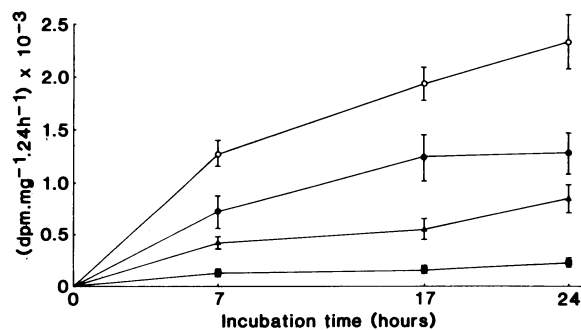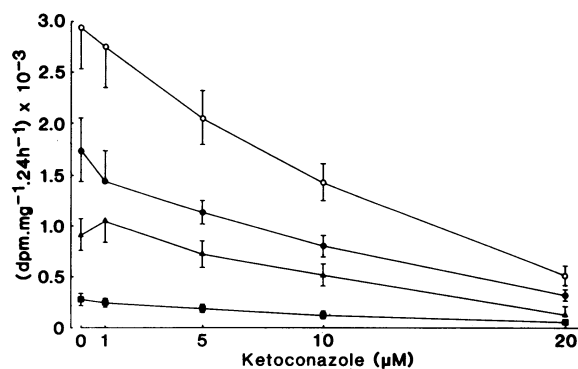
Figure 1. Time course of bile acid synthesis in rat hepatocytes. Hepatocytes were isolated and cultured as described in Methods. 4 h after cell seeding in 10 cm^2-wells 0.15 μCi [4-^{14}C]cholesterol in culture medium was added. Cells and medium were harvested at indicated times, cells were washed four times after removal of medium and bile acids were determined. ●, cholic acid; ▲, β-muricholic acid; ■, chenodeoxycholic acid; and ○, total bile acids. The values shown represent the sum of intracellular and extracellular bile acids and are means (±SD) of four experiments with duplicate incubations.

Figure 2. Inhibition of bile acid synthesis in rat hepatocytes by ketoconazole. 4 h after plating in 100-mm diameter tissue culture dishes, 1 μCi [4-^{14}C]cholesterol in culture medium and 25 μl of ketoconazole, dissolved in 50 mM HCl, was added. After 24 h incubation radioactivity in intracellular and extracellular bile acids was determined. Symbols are as described in Fig. 1. Values shown are means (±SD) of five experiments with duplicate incubations.
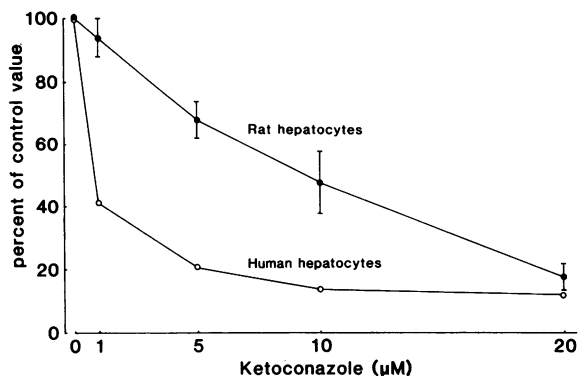
Figure 3. Effect of ketoconazole on bile acid synthesis by human and rat hepatocytes. Human hepatocytes were isolated and cultured as described in Methods. Inhibition by ketoconazole of cholic acid and chenodeoxycholic acid synthesis by human hepatocytes was measured during a 24-h incubation from 42 to 66 h after plating on 100-mm diam tissue culture dishes. Ketoconazole and [4-^{14}C]cholesterol were added as described in legend of Fig. 2. Radioactivity in bile acids was determined in medium and cells. Intracellular bile acids comprised ~15% of total bile acids. Absolute synthesis of cholic and chenodeoxycholic acids in medium and cells without ketoconazole (100% value) was 2195±263 dpm·mg^{-1}·24 h^{-1}, whereas cholic acid-chenodeoxycholic acid ratio was 55:45. For comparison values obtained from experiments with rat hepatocytes (Fig. 2) are shown.

acid synthesis from [^{14}C]7α-hydroxycholesterol even at 20 μM ketoconazole (see for comparison Fig. 3 and Table I).

Effect of ketoconazole on liver microsomal cholesterol 7α-hydroxylase activity. The above-mentioned experiments with rat hepatocyte monolayer cultures suggest that inhibition of bile acid synthesis by this antimycotic agent takes place at the first and rate-limiting enzyme in bile acid synthesis pathway, cholesterol 7α-hydroxylase. To confirm this finding, we incubated ketoconazole with microsomes prepared from rat liver. Addition of various concentrations of ketoconazole to the reaction mixture resulted in a strong inhibition of cholesterol 7α-hydroxylase activity, giving 34, 75, and 94% inhibition at 0.2, 2, and 10 μM ketoconazole, respectively, which was significantly different (*P* < 0.05) from the control value without ketoconazole (1.28±0.10 nmol·mg^{-1}·h^{-1}). A linear relationship between logarithmic concentration of the drug and inhibition of cholesterol 7α-

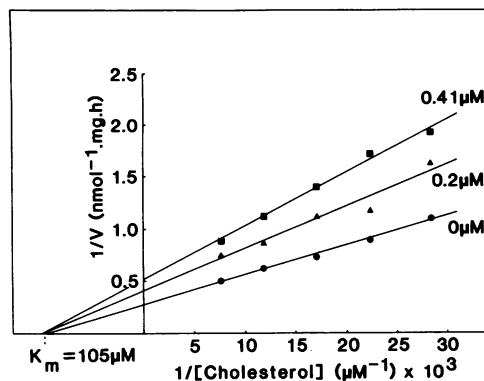

Figure 4. Lineweaver-Burk plots showing the inhibitory effects of ketoconazole on cholesterol 7α-hydroxylase activity. Incubations were performed in the presence of 0.41 μM (■), 0.20 μM (▲) or no (●) ketoconazole. The figure shown is representative for three independent experiments with liver microsomes isolated from two rats.

hydroxylase was found, giving an I$_{50}$-value of 0.4 μM. Kinetic studies were performed to determine the mechanism of inhibition of cholesterol 7α-hydroxylase. As shown in Fig. 4, the fact that the apparent K_m-value for the enzyme (105 μM) was virtually unchanged in the presence of ketoconazole demonstrates noncompetitive inhibition. The Dixon plot in Fig. 5 confirms this finding, giving a K_i-value for ketoconazole of 0.4 μM, comparable to the I$_{50}$-value obtained with these microsomes and much lower than the apparent K_m for cholesterol, indicating that the drug has a strong affinity for the enzyme.

Effect of ketoconazole on biliary excretion of bile acids and cholesterol, and on bile flow in vivo. In vivo experiments were performed in rats to validate our findings obtained in monolayers of human and rat hepatocytes by ketoconazole. Rats with chronic total bile diversion (18) were used, since in these animals the bile acid pool is depleted and bile acid synthesis can be measured as biliary bile acid excretion. As demonstrated in Fig. 6 during long-term interruption of the enterohepatic circulation, both bile flow and biliary bile acid excretion displayed clear circadian variations, in agreement with a previous report (17). Administration of 50 mg/kg ketoconazole via the duodenal catheter led
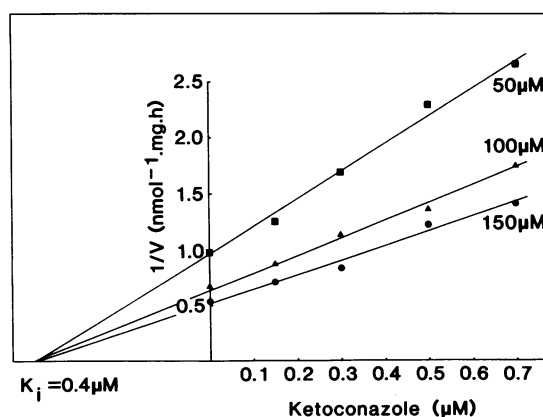
Table I. Effect of Ketoconazole on Conversion of 7α-Hydroxycholesterol into Bile Acids by Rat Hepatocytes

Ketoconazole	Conversion of 7α-hydroxycholesterol
μM	%
0	91±2
5	89±4
20	85±4

Hepatocytes were prepared and seeded as described in Methods. [4-^{14}C]7α-hydroxycholesterol 6 × 10^3 dpm/well together with fresh medium was added to 10 cm^2-wells at 4 h after plating. Cells were incubated for 21 h, medium and hepatocytes were harvested and extracted according to Bligh and Dyer (19). After phase separation, radioactivity in the methanol-water phase was determined and expressed as percentage conversion of total recovered disintegrations per minute. Values shown are means (±SD) of four experiments with duplicate incubations.

Figure 5. Dixon plots of the inhibition of cholesterol 7α-hydroxylase by ketoconazole. Substrate concentrations were 50 μM (■), 100 μM (▲), and 150 μM (●) cholesterol. The figure shown is representative for three independent experiments with liver microsomes isolated from two rats.
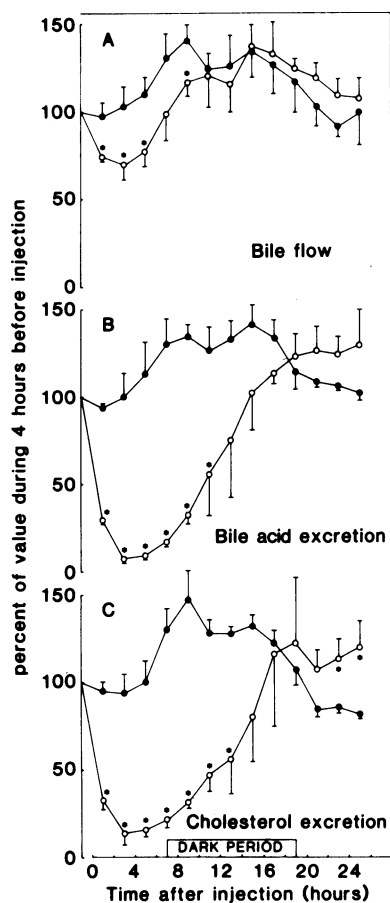
Figure 6. Effects of ketoconazole on bile flow (A) and excretion rates of bile acids (B) and cholesterol (C) in bile-diverted rats. Rats were given 50 mM HCl (●) or a solution of ketoconazole (50 mg/kg body weight) in 50 mM HCl (O) via the duodenal catheter at t = 0 (12 a.m.). Bile collection was started 4 h before t = 0. Rates for the 26 h after t = 0 were expressed as percentage of their values in the last 4 h prior to injection, for each individual experiment. The values shown represent means (±SD) of these percent values of experiments with three animals per group. A significant difference (P < 0.05) between ketoconazole-treated and control rats is indicated by an asterisk. Absolute values in the 4 h before injection (means±SD) are

| | | Excretion | |
	Bile flow	Bile acids	Cholesterol
	$(ml \cdot h^{-1})$	$(\mu mol \cdot h^{-1})$	$(\mu mol \cdot h^{-1})$
Control	0.42±0.05	11.3±3.4	0.073±0.003
Ketoconazole	0.49±0.02	11.6±1.5	0.070±0.003

to a rapid decrease in bile flow, reaching its minimum value after 2 to 4 h (Fig. 6 A). Bile acid and cholesterol excretion concomitantly declined dramatically to 8 and 15%, respectively, of the baseline value in the last 4 h before injection (Fig. 6 B and C). Since in these bile-diverted animals bile acid output represents bile acid synthesis, we conclude that ketoconazole blocked synthesis of bile acids almost completely.

A similar inhibition of bile acid excretion in bile-diverted rats was observed at much lower doses of 5 and 10 mg/kg, resulting in a significant decrease of biliary bile acid excretion to

63 and 32% of the control value after 2 to 4 h, respectively. This reduction continued for a shorter time as compared with the effect found after 50 mg/kg (data not shown).

In order to check whether this fall in bile acid excretion was accomplished by a reduced supply of cholesterol by inhibition of cholesterol synthesis, we injected rat serum labeled with [4-^{14}C]cholesterol intracardially in ketoconazole-treated and control rats 2 h after administration of ketoconazole and solvent, respectively. No difference was observed in the distribution of radioactivity and disappearance from plasma in both groups (data not shown). However, ketoconazole inhibited excretion of ^{14}C-radioactivity in bile during the period in which bile acid production was blocked (Fig. 7). Because more than 97% of total ^{14}C-radioactivity is excreted as bile acids (Table II), inhibition of excretion of ^{14}C-radioactivity must be caused by a diminished bile acid synthesis.

In order to test whether ketoconazole depressed bile acid output by blocking their transport across the canalicular membrane, a tracer dose of [^{14}C]taurocholate was given intracardially 3 h after ketoconazole (50 mg/kg) administration. Bile was collected and radioactivity in bile determined. Recovery of ^{14}C-radioactivity in bile 1 h after injection was 89.3±3.2% for control rats and 93.5±5.4% for drug-treated animals. This finding demonstrates that bile acid transport across both sinusoidal and canalicular membranes of hepatocytes is not appreciably hampered by ketoconazole.

Discussion

Monolayer cultures of rat hepatocytes were shown to synthesize bile acids from labeled precursor in a linear fashion between 4 and 28 h after plating (the period in which the effects of ketoconazole were studied, Fig. 1). Cholic acid comprised 55 to 65% of total synthesized bile acids during this time interval, a value in good agreement with cholic acid content in vivo (38, 39). On the other hand the ratio of β-muricholic to chenodeoxycholic acid was slightly increased as compared with the value in bile of whole animals (40). Similar cholic acid content in rat hepatocyte monolayer cultures was reported by Davis et al. (20). These authors, however, did not detect chenodeoxycholic acid,
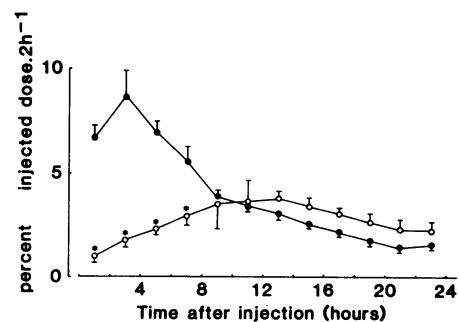

Figure 7. Effect of ketoconazole on biliary excretion of ^{14}C-radioactivity after injection of serum-associated [4-^{14}C]cholesterol. 2 h after administration of 50 mM HCl (control, ●) or 50 mg/kg ketoconazole (o) 0.75 ml rat serum labeled with [4-^{14}C]cholesterol (0.5 μCi/rat) was injected via the heart catheter. Bile was collected and radioactivity in an aliquot was determined. Values are expressed as percentage of the injected dose and are means (±SD) of experiments with three rats per group. A significant difference (P < 0.05) between control and ketoconazole-treated animals is indicated by asterisks.

Table II. *Recovery of* ^{14}C-*Radioactivity in the Bile Acid and Cholesterol Fraction of Bile from Ketoconazole-treated and Control Rats*

	Recovery of ^{14}C-radioactivity as percentage of injected dose					
	Time after injection 12 h			24 h		
Experimental group	Bile acids	Cholesterol	Total	Bile acids	Cholesterol	Total
Control	35.2±3.4	1.2±0.2	36.7±2.9	49.3±1.6	1.6±0.4	51.0±1.8
Ketoconazole	15.1±2.0*	0.5±0.1*	15.6±2.0*	32.6±1.8*	1.1±0.1	33.4±1.9*

2 h after administration of solvent (control) or ketoconazole (50 mg/kg) rat serum labeled with [4-^{14}C]cholesterol was injected via the heart catheter into both groups. Bile was collected, an aliquot was extracted and radioactivity was determined in bile, and chloroform and methanol-water phase after extraction. Values shown are means (±SD) of experiments with three rats per group.   * Significant difference ($P < 0.05$) between control and ketoconazole-treated animals.

which was found to represent 10 to 15% of total bile acid synthesis by rat hepatocytes (41, 42). It has been suggested, that culture medium which contains insulin, as used by Davis et al. and in our experiments (135 nM), is responsible for the high cholic acid content by stimulating the 7α-hydroxy-cholest-4-en-3-one 12α-hydroxylase (42). This enzyme is thought to control the ratio of cholic acid to chenodeoxycholic plus β-muricholic acid (43). Approximately 75% of synthesized bile acids was found intracellularly during the 4- to 28-h incubation, but no specific retention of any individual bile acid was observed. This observation is in contrast with Hylemon et al. (37), who found 20% of total bile acids to be associated with the cells after 24 h. In human hepatocyte monolayer cultures, in which the effect of ketoconazole on bile acid synthesis was measured from 42 to 66 h after cell seeding, intracellular bile acids comprised 15% of total bile acids. A similar value was observed by us with rat hepatocytes 48 to 72 h in culture (data not shown) and by others (20). This finding indicates that intracellular concentrations of bile acids in hepatocytes decrease with time.

The high amount of bile acids present in the hepatocytes during the initial 24 h may be the result of an impaired excretion or an increased cellular uptake by the rat hepatocytes. Cultured hepatocytes are able to accumulate high intracellular concentrations of bile acids from the medium (25, 44). It is interesting that in presence of 20 μM ketoconazole the cell-associated bile acids were significantly lower than in control incubations without the drug. It is tempting to speculate that uptake of bile acids at this concentration of ketoconazole is impaired due to changes of the cell membrane, provoked either by a direct interaction of ketoconazole with membrane lipids or indirectly via the effect of increased lanosterol on membrane and cell functions (3, 45).

This paper demonstrates for the first time that human hepatocytes in monolayer culture synthesize cholic and chenodeoxycholic acid. Since we show results from only one hepatocyte isolation, due to the limited availability of human liver, no conclusions should be drawn concerning the absolute synthesis rate of these bile acids. However, it is obvious from this experiment that ketoconazole blocks bile acid synthesis in human hepatocytes in a similar way as observed with rat hepatocytes.

Production of bile acids was inhibited in monolayer cultures of rat and human hepatocytes (Fig. 2 and 3, I_{50} was 5 μg/ml and 0.5 μg/ml, respectively), at concentrations within the therapeutic plasma level of the drug (2–20 μg/ml) used in patients (46). These I_{50}-values for bile acid synthesis in hepatocytes are of a same magnitude as concentrations inhibiting 50% corticosterone

production in rat adrenal cells (10) and 25-hydroxy-vitamin D-24-hydroxylase in pig kidney cells (11).

In agreement with others (37) we showed that [4-^{14}C]7α-hydroxycholesterol is converted to bile acids (Table I). Since this conversion was almost complete and not blocked by ketoconazole, this is direct evidence that inhibition of bile acid synthesis happened at the cholesterol 7α-hydroxylase enzyme. No evidence was obtained that other enzymes in bile acid synthesis pathway, i.e., the 12α-hydroxylase and the 6β-hydroxylase were blocked, since ratios of cholic acid to β-muricholic acid plus chenodeoxycholic acid, and of chenodeoxycholic acid to β-muricholic acid did not change with increasing amounts of ketoconazole (Fig. 2). This finding together with results from other studies (9, 11, 13, 45) suggests that the effect of ketoconazole on cytochrome P-450-mediated oxidative drug metabolism may be selective. For example, it appears that fungal 14α-demethylase is inhibited at a 140 times lower concentration than cholesterol synthesis in human fibroblasts (45) and that testosterone synthesis is blocked selectively at the C_{17-20}-lyase (9).

In vivo experiments in bile-diverted rats confirmed the in vitro observations on inhibition of bile acid synthesis by ketoconazole (Figs. 6 and 7). A dramatic decrease in biliary bile acid excretion to 8% of the control value was found. This reduction was not due to a blockade of transport of bile acids from liver cells into bile, since excretion of labeled taurocholate in bile was not impaired by the drug.

Another explanation for the declined bile acid excretion in vivo might be a decreased supply of substrate for bile acid synthesis. This possibility had to be considered, since in rats with a chronic bile drainage newly made cholesterol is a major substrate for bile acid formation (47–49) and ketoconazole blocks cholesterol synthesis at the level of 14α-demethylation (2, 3). On the other hand, it is known that, especially at low bile acid excretion rates, there is a strong correlation between biliary cholesterol and bile acid excretion (50, 51), and that cholesterol excretion in the rat does not critically depend on the rate of hepatic cholesterogenesis (52). In addition, we demonstrated that conversion of labeled cholesterol into bile acids is almost complete and inhibited by ketoconazole (Fig. 7 and Table II), indicating that bile acid production is blocked in vivo. The impaired biliary cholesterol output, therefore, is probably secondary to a decreased biliary bile acid excretion.

The K_i of cholesterol 7α-hydroxylase for ketoconazole is considerably lower than the K_i-value found with metyrapone (0.55 mM), another noncompetitive inhibitor of cholesterol 7α-

hydroxylase (53). Metyrapone is a drug with a cytochrome P-450 blocking activity, used to treat hyperadrenocorticosteroidism. Since it has been suggested to exploit ketoconazole for a similar purpose (10, 11), this difference in potency could mean that ketoconazole is a preferable alternative in this treatment.

In summary, the data presented in this paper indicate that ketoconazole very effectively inhibits bile acid synthesis in vivo and in vitro not merely by blocking cholesterogenesis (2–5), but by a direct effect on the regulatory step in the catabolism of cholesterol to bile acids, cholesterol 7α-hydroxylase. In the light of the inhibited cholesterogenesis and bile acid synthesis, it would be interesting to determine the effect of ketoconazole on biliary excretion of bile acids and lipids and on size of the bile acid pool and bile composition in humans treated with this drug. Long-term treatment of patients with ketoconazole may lead to a reduced bile acid pool size, resulting in an impaired absorption of lipid-soluble nutrients, e.g., vitamins A, D, K, and E.

The fact that this antimycotic agent strongly blocks bile acid synthesis at concentrations easily achieved in plasma of patients treated for fungal infections (2–20 µg/ml) (46), may be usefully applied in clinical practice, in which it would be desirable to inhibit bile acid synthesis, e.g., intractable pruritus due to cholestasis in primary biliary cirrhosis or with extrahepatic obstruction (28). Furthermore, our finding regarding the actions of ketoconazole on cytochrome P-450–dependent enzymes involved in bile acid synthesis, add evidence to the thesis that ketoconazole is a selective inhibitor of cytochrome P-450-activity (13, 45). This information warrants further investigation of the drug in clinical research to detect specific effects on other cytochrome P-450–dependent metabolic pathways.

Acknowledgments

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