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EFFECT OF KETOCONAZOLE ON CHOLESTEROL SYNTHESIS AND ON HMG-COA REDUCTASE AND LDL-RECEPTOR ACTIVITIES IN HEP G2 CELLS

HERMAN JAN KEMPEN,* KEES VAN SON, LOUIS H. COHEN, MARIEKE GRIFFIOEN, HANS VERBOOM and LOUIS HAVEKES

TNO Gaubius Institute for Cardiovascular Research, Herenstraat 5d, 2313 AD Leiden, The Netherlands

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Abstract—Ketoconazole, an imidazole derivative, is a member of a class of metabolic inhibitors acting specifically at cytochrome-P450 mediated reactions. We studied the effects of this compound on cholesterol synthesis, and on HMG-CoA reductase and LDL receptor activities, in cultures of human hepatoma cell line Hep G2.

Ketoconazole, added in concentrations of 2–100 μM, inhibited cholesterol synthesis, and caused accumulation of lanosterol and dihydrolanosterol. Total mass formation of sterols was depressed. After 20 hr preincubation of the cells with the drug in these concentrations, activity of HMG-CoA reductase was markedly decreased, while the receptor-mediated binding, uptake and degradation of human LDL were increased. This increase is at least partly due to a higher affinity of LDL for its receptor. Ketoconazole prevented the fall in LDL–receptor activity caused by preincubation with LDL, whereas it did not affect the suppression caused by preincubation with exogenous mevalonate.

These findings are discussed with respect to the involvement of endogenous sterol and non-sterol effectors of reductase and receptor activities.

Ketoconazole (*cis*-1-acetyl-4-(4-(2-(2,4-dichlorophenyl)-2-(1-H-imidazol-1-ylmethyl-1,3-dioxalan)-methoxy)phenyl)piperazine) is an orally active imidazole antimycotic agent, effective against a wide range of fungal pathogens [1]. The drug inhibits the conversion of lanosterol to ergosterol in fungal organisms at low (nanomolar) concentrations, and to cholesterol in mammalian cells at much higher (micromolar) concentrations. Its mode of action in both cases consists in blockade of the cytochrome-P450 mediated C14-demethylation of lanosterol [2]. Recently, ketoconazole has been shown to inhibit other cytochrome-P450 dependent steroidogenic reactions in adrenal [3, 4] and gonadal [5, 6] tissues, and cholesterol- [7] and drug-metabolizing activities in rat liver [8, 9].

We were interested in this compound from the standpoint of metabolic regulation. For both HMG-CoA† reductase and LDL–receptor activities, a suppressive role of LDL-cholesterol has been demonstrated in liver and extrahepatic cells [10–14]. In addition, repression of these activities is achieved by endogenous mevalonate-derived products since both reductase and LDL–receptor are induced after incubation of cultured cells with compactin or mevinolin, specific blockers of mevalonate synthesis [11–15]. For the reductase, this induction could be prevented completely by addition of mevalonate [12, 13, 15], but not by LDL [12, 14, 15], suggesting that a mevalonate metabolite other than cholesterol acts as endogenous repressor. For the receptor, the induction by compactin could be counteracted entirely by LDL as well as by mevalonate [13], suggesting that cholesterol is the major repressor for this protein.

We reasoned that experiments with ketoconazole, blocking cholesterol synthesis at the level of lanosterol conversion, might enable us to decide to what extent endogenously synthesized sterols derived from lanosterol play a role as endogenous repressor(s) of reductase or receptor activity.

In this paper we confirm the blocking action of ketoconazole at lanosterol demethylation, and report a decreased HMG-CoA reductase activity and an increased receptor-mediated cellular association and degradation of LDL, after incubation of Hep G2 cells with ketoconazole.

EXPERIMENTAL

Materials. Ketoconazole was a kind gift of Dr H. van den Bossche, Janssen Pharmaceutica, Beerse, Belgium. It was dissolved in 0.05 M HCl, and diluted to the desired concentration in DMEM medium. Control incubations were run containing the same amount of HCl.

Methods. Hep G2 cells were cultured in DMEM containing 10% FCS, in multi-well dishes [16] and used when grown at a density of 50–100 μg cell protein per cm^2, 3–5 days after seeding. In order to study the effects of ketoconazole, the cells were washed and incubated for 20 hr in DMEM containing 1% HSA and ketoconazole in concentrations specified under Results.

In experiments aimed at documenting the effect on sterols synthesis, the medium contained 0.1 μCi [2-^{14}C]-acetate during this 20 hr incubation period. At the end of this period, the medium was aspirated, the cells were washed with 0.15 ml of buffer con-

* To whom all correspondence should be addressed.

† Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DMEM, Dulbecco's modification of minimal essential medium; FCS, fetal calf serum; NSL, non-saponifiable lipids; LDL, low density lipoprotein.

taining 100 mM K_2HPO_4/KH_2PO_4, 100 ml NaCl and 10 mM Na_2EDTA (pH 7.4) (KNE-buffer) whereafter the wash-fluid was combined with the medium. The cells were then dissolved in 0.3 M NaOH. After taking an aliquot for protein determination, the homogenate was combined with the media, and total lipids were extracted [17].

The lipids were saponified in 0.5 M KOH in 96% ethanol for 90 min at 60°, and after addition of an equal volume of water the non-saponifiable lipids (NSL) were isolated by hexane extraction. Hexane was removed by evaporation under a stream of N_2, and the NSL were separated by thin-layer chromatography on silica plates, using a mixture of hexane and acetone (8/2, by vol.) as developing solvent. The plate was then autoradiographed after spraying with Enhance-R, and the zones containing radioactive lipids scraped into scintillation vials and mixed with Instafluor. These zones were identified as to contain polar sterols (R_f 0.35–0.40), cholesterol (R_f 0.44) and lanosterol or dihydrolanosterol (both with R_f 0.56) by using cholesterol and a mixture of lanosterol and dihydrolanosterol as reference markers.

For determination of the effect of the drug on the total mass of various sterols the same experiments were carried without adding labeled acetate. Campesterol was added as internal standard before lipid extraction, and aliquots of the NSL-fraction in hexane were directly subjected to gas–liquid chromatography, using a capillary column coated with CP-Sil-5, run at 230° using hydrogen as carrier gas.

In order to assess the effect on total HMG-CoA reductase activity, the same protocol was followed as described before [13]. The cells were preincubated for 20 hr in DMEM containing 1% HSA with increasing ketoconazole concentrations whereafter the medium was removed and the cells scraped and sonicated in KNE-buffer for 5 sec. To determine the changes in LDL metabolism caused by the preincubations with ketoconazole, receptor-mediated binding, uptake and degradation of [^{125}I]-LDL by cells at 37° were monitored as described in [16], at concentrations of up to 50 μg/ml.

Differences were considered to be statistically significant when $P < 0.05$ using Student's t-test.

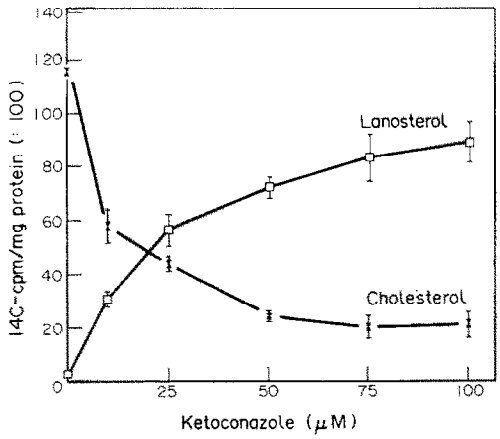

Fig. 1. Effect of increasing concentrations of ketoconazole on the incorporation of [2-^{14}C]-acetate in sterols in Hep G2 cells during 20 hr incubation. Symbols represent means ± SD of three separate experiments.

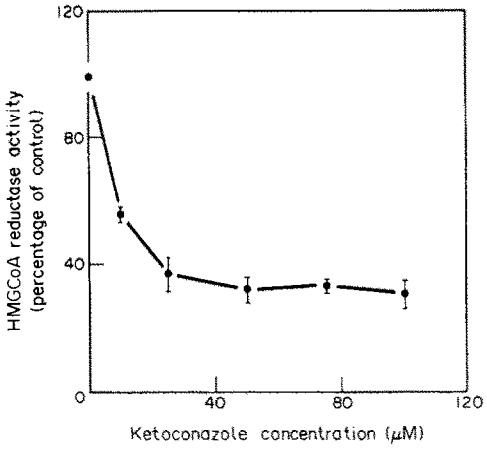

Fig. 2. Effect of preincubation of Hep G2 cells with increasing concentrations of ketoconazole for 20 hr on the HMG-CoA reductase activity. Data are expressed as % of the control value in the absence of the drug; symbols represent means ± SD for three separate experiments.

RESULTS

After control incubations of Hep G2 cells with [^{14}C]-acetate more than 90% of all radioactivity in the NSL fraction chromatographed in a spot with the R_f-value of cholesterol. In the incubations with 10–100 μM ketoconazole, label in cholesterol decreased and radioactivity appeared in a spot with the R_f of lanosterol (Fig. 1). This effect appeared to be maximal at 100 μM of the drug. A small percentage of label (15 ± 7% of total radioactivity in NSL) occurred in the form of compounds more polar than cholesterol in all incubations; this percentage was independent of the drug concentration.

Incubation of the cells for 20 hr with increasing concentrations of ketoconazole caused a fall in the total HMG-CoA reductase activity as determined in the cell sonicate (Fig. 2). The maximal effect was reached at 25 μM ketoconazole. The drug had no effect on the activity of the enzyme when directly added to the assay mixture in concentrations up to 1 mM (not shown).

In order to see whether this fall in HMG-CoA reductase activity by ketoconazole was accompanied by an absolute decrease in the rate of sterols synthesis, the mass increments of the various non-saponifiable lipids arising during incubation without or with ketoconazole were determined by quantitative gas-chromatography. Cholesterol was the only sterol seen to accumulate in the control incubation. In the presence of increasing concentrations of ketoconazole the increment in cholesterol was markedly lower, while lanosterol and dihydrolanosterol were found in detectable amounts (Fig. 3). However, the drug clearly lowered the total mass of sterol synthesized during 24 hr incubation.

Pretreatment of the cells with ketoconazole for 20 hr increased the association (binding plus uptake)

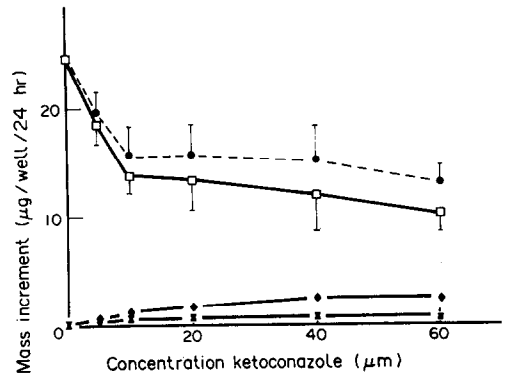

Fig. 3. Mass increments of cholesterol (□), lanosterol (◆) and dihydrolanosterol (X), and of total sterols (●) during 24 hr incubation of Hep G2 cells in the presence of 0, 5, 10, 20, 40 or 60 μM ketoconazole. Means of six wells for each condition. Vertical lines indicate half the SD.

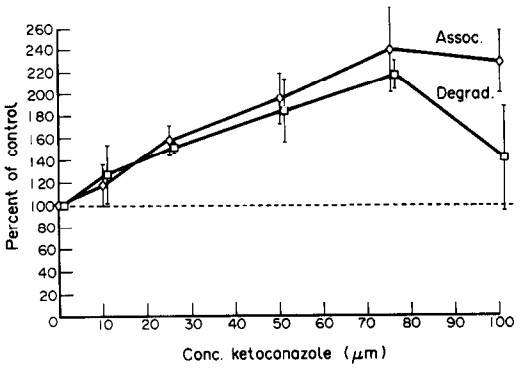

Fig. 4. Effect of preincubation of Hep G2 cells with increasing concentrations of ketoconazole for 20 hr on the receptor-mediated association (binding plus uptake) and degradation of 10 μg/ml ^{125}I-LDL. Data are expressed as % of control value without drug; symbols represent means ± SD of four separate experiments.

and degradation of 10 μg/ml [^{125}I]-LDL (Fig. 4). The stimulatory effect was maximal at 75 μM. In order to document this drug effect in more detail, the effect of 75 μM ketoconazole on binding and uptake was studied with concentrations of labeled LDL varying between 2 and 50 μg/ml. As shown in Fig. 5, both binding and uptake are increased significantly over this concentration range. When the binding data were analyzed by the method of Scatchard (Fig. 6), the association constant was found to be 268 ± 126% (N = 4) higher in the ketoconazole treated cells, whereas there was no significant change of the maximal number of binding sites. The stimulation of LDL-association by ketoconazole was still observed when up to 10 μM cycloheximide was also present during the preincubation (not shown).

Finally, the question was addressed whether the increase in receptor-mediated LDL-association was secondary to cholesterol depletion or decreased mevalonate formation, occurring in the presence of ketoconazole. Therefore, the effects of this drug on the LDL-association were also studied in the

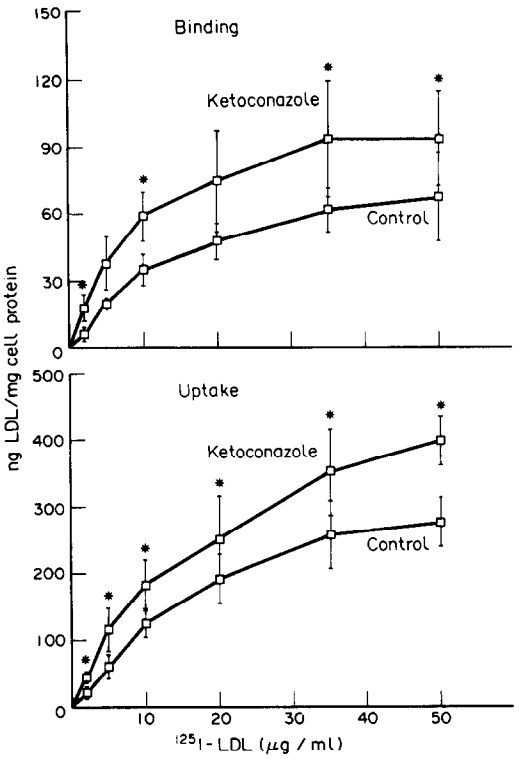

Fig. 5. Effect of preincubation of Hep G2 cells with 75 μM ketoconazole for 20 hr on the receptor-mediated binding and uptake of 2–50 μg/ml ^{125}I-LDL. Symbols represent means ± SD of four separate experiments. The stimulation by ketoconazole was statistically significant for the LDL concentrations indicated by an asteristk.

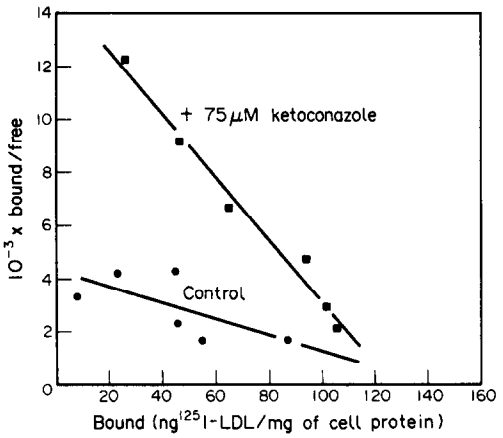

Fig. 6. Effect of preincubation of Hep G2 cells with 75 μM ketoconazole for 20 hr on the binding of ^{125}I-LDL. Data of a single experiment are plotted according to the method of Scatchard. Similar plots were obtained in three other experiments.

presence of LDL (200 μg protein/ml) or mevalonate (10 mM), added simultaneously. Ketoconazole could counteract completely the suppressive effect of LDL on the receptor-mediated LDL-association whereas the suppression by exogenous mevalonate (about 70%) was not affected by the drug (Table 1).

Table 1. Effect of preincubation with 10 mM mevalonate (MVA) or 200 μg/ml human LDL, alone or together with ketoconazole (75 μ), on receptor-mediated association of 10 μg/ml [^{125}I]-LDL by Hep G2 cells at 37°

	Control	Ketoconazole
	% of control	
no MVA or LDL	100	233 ± 16[a]
LDL	56 ± 3[b]	208 ± 25[a]
MVA	34 ± 8[b]	89 ± 5[a,b]

Means ± SD of 3 separate experiments.
[a] Significantly different from value in left column.
[b] Significantly different from value in upper row.

DISCUSSION

In this paper we report effects of ketoconazole on synthesis of sterols, on HMG-CoA reductase activity and on receptor-mediated LDL-metabolism in Hep G2 cells. As stated in the Introduction, this drug is known to interfere with cholesterol synthesis in mammalian organisms at the level of lanosterol C14-demethylation. This action is confirmed in the present study with a human hepatoma cell line.

It has been demonstrated by several laboratories that the amount of HMG-CoA reductase protein and activity rises after preincubation with compactin, but that this rise can be prevented by co-addition of mevalonate [12–15]. These findings have led to the conclusion that synthesis of the reductase enzyme is normally kept suppressed by mevalonate or a mevalonate-derived metabolite, which may be a sterol or non-sterol compound. The present observation that reductase activity is decreased rather than increased after incubation of the cells with ketoconazole, suggests that (an) endogenous suppressor molecule(s) can be formed and even may accumulate in the presence of this drug. This suppressor might be lanosterol itself or a lanosterol derivative (whose formation then should not require C14-demethylation). Earlier, Berg *et al.* [18] also observed a marked decrease in HMG-CoA reductase activity after treatment of yeast cells with the compounds clotrimazole and triadimefon, which are related in structure and activity to ketoconazole. The effect of these drugs could be mimicked by addition of exogenous lanosterol. Neither these compounds nor lanosterol had a direct inhibitory effect on the reductase in isolated yeast microsomes. Our findings with ketoconazole (this paper) and with buthiobate [19], another blocker of sterol synthesis claimed to act specifically at the C14-demethylation step, extend the relation between lanosterol accumulation and decreased reductase activity to human liver cells.

Recently, Gupta *et al.* [20] reported a biphasic response in reductase activity upon treatment of rat intestinal epithelial cells with ketoconazole. The enzyme activity was decreased by concentration below 15 μM, but stimulated by 30 μM of the drug. Since lanosterol accumulated also at this high concentration, they concluded that lanosterol itself is not able to suppress the reductase activity. Their data furthermore indicated the existence of a regulatory polar sterol, derived from 24,25-epoxylanosterol through a cytochrome P-450 dependent reaction, which would be blocked only by higher concentrations of ketoconazole. In contrast, in our studies with Hep-G2 cells ketoconazole and buthiobate decreased HMG-CoA reductase and total sterols synthesis in concentrations of up to 100 μM. The reason for this discrepancy is unclear at this moment, and will be the subject of further study in our laboratory.

Interestingly, we observed an increase in receptor-mediated binding, uptake and degradation of iodinated LDL by the Hep G2 cells. This suggests that either cholesterol or one of its precursors derived from lanosterol normally plays a restrictive role in receptor-mediated LDL-uptake. The findings that (i) the increased binding was due to a higher binding affinity, and that (ii) the increased association still occurred in the presence of cycloheximide, suggest that no new receptor synthesis is involved, and that the effect on LDL-binding and uptake may be secondary to conformational changes in the LDL-receptor molecule, induced by the accumulated lanosterol/dihydrolanosterol, or by the drug itself [21]. The latter possibility should not be entirely excluded in spite of the report [22] that ketoconazole causes no gross disturbances in the organization of artificial lipid bilayers.

The fall in LDL-association caused by pre-incubation of the cells with LDL could be prevented by simultaneous addition of ketoconazole. The mechanism of this preventive effect is as yet unclear; the drug might physically prevent LDL-cholesterol from reaching the site of its suppressive action, or it might inhibit a putative enzyme(s) (cytochrome P450 dependent?), converting this cholesterol into an effective suppressor of the receptor. In line with this observation, Gupta *et al.* [20] reported that ketoconazole could block the suppressive effect of LDL on HMG-CoA reductase in intestinal epithelial cells. They suggest that the drug may inhibit the cytochrome-P450 dependent (LDL-induced) formation of an endogenous oxysterol. We submit that the drug may as well inhibit the conversion of exogenous (LDL-) cholesterol into a regulatory effective oxysterol. As regards the stimulation of the receptor activity by ketoconazole, it can be concluded that this effect is not secondary to depletion of cholesterol, since the stimulation occurs to the same extent in the presence of exogenous (LDL-) cholesterol. Furthermore, ketoconazole could partially prevent the decrease in receptor activity caused by mevalonate. In fact, the drug stimulated the receptor activity relatively to the same extent in the presence of added mevalonate as in its absence. This suggests that the receptor stimulation by ketoconazole also was not secondary to the fall in endogenous mevalonate formation.

In two recent papers [23, 24] a fall in the serum cholesterol level is reported in patients undergoing antifungal treatment with this drug. This may be expected in the light of the inhibited reductase and increased receptor-mediated LDL-uptake by human Hep G2 cells, described in this paper.

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