Differential regulation of low density lipoprotein suppression of HMG-CoA reductase activity in cultured cells by inhibitors of cholesterol biosynthesis[1]

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Abstract Treatment of rat intestinal epithelial cells (IEC-6 cells) with lanosterol 14α-demethylase inhibitors, ketoconazole and miconazole, had similar effects on 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity and cholesterol biosynthesis but the drugs differed in their ability to prevent the low density lipoprotein (LDL) suppression of reductase activity. Miconazole, at concentrations that inhibited the metabolism of lanosterol and epoxylanosterol to the same degree as ketoconazole, did not prevent low density lipoprotein action on reductase activity, whereas ketoconazole totally abolished the low density lipoprotein action on reductase activity. Both drugs caused: *1*) a biphasic response in reductase activity such that at low concentrations (<2 μM) reductase activity was inhibited and at high concentrations (>5 μM) the activity returned to control or higher than control levels; *2*) an inhibition of metabolism of lanosterol to cholesterol, and 24(S), 25-epoxylanosterol to 24(S), 25-epoxycholesterol. Neither drug prevented suppression of reductase activity by 25-hydroxylanosterol, 25-hydroxycholesterol, or mevalonolactone added to the medium. Each drug increased the binding, uptake, and degradation of ^{125}I-labeled LDL and inhibited the re-esterification of free cholesterol to cholesteryl oleate and cholesteryl palmitate. The release of free cholesterol from [^{3}H]cholesteryl linoleate LDL could not account for the differential effect of ketoconazole and miconazole on the prevention of low density lipoprotein suppression of reductase activity. The differential effect of the drugs on low density lipoprotien suppression of reductase activity was not unique to IEC-6 cells, but was also observed in several cell lines of different tissue origin such as human skin fibroblast cells (GM-43), human hepatoblastoma cells (HepG2), and Chinese hamster ovary cells (wild type, K-1; 4α-methyl sterol oxidase mutant, 215). These observations suggest that the suppressive action of low density lipoprotein on reductase activity *1*) does not require the de novo synthesis of cholesterol, or 24(S), 25-epoxysterols; *2*) is not mediated via the same mechanism as that of mevalonolactone; and *3*) does not involve cholesteryl re-esterification. Ketoconazole blocks a site in the process of LDL suppression of reductase activity that is not affected by miconazole. —**Gupta, A. K., R. C. Sexton, and H. Rudney.** Differential regulation of low density lipoprotein suppression of HMG-CoA reductase activity in cultured cells by inhibitors of cholesterol biosynthesis. *J. Lipid Res.* 1990. **31:** 203–215.

Supplementary key words low density lipoprotein • ketoconazole • miconazole • epoxysterol metabolism • oxysterols

Low density lipoprotein (LDL) regulates the biosynthesis of cholesterol in cultured cells by inhibiting the activity of HMG-CoA reductase, one of the key regulatory enzymes in cholesterol biosynthesis (*1*). It has been suggested that LDL action on reductase activity may be mediated by the formation of oxysterol(s) which can suppress the activity of HMG-CoA reductase (2–9). Oxysterol(s) can be formed either from lanosterol, an obligatory precursor of cholesterol, or from diversion of squalene 2:3-epoxide to squalene 2:3,22:23-dioxide and subsequent cyclization to epoxysterols, or from the controlled enzymic oxidation of endogenous cholesterol or exogenous cholesterol derived from LDL (5, 6).

Formation of oxysterols by the above pathways has been shown to involve cytochrome P450-dependent reactions (5, 9–11). Evidence has also been presented that shows that several inhibitors of cytochrome P450-dependent reactions abolish the action of LDL on reductase activity without significantly interfering with LDL processing (5, 9, 10). We have recently reported (11) that LDL added to the medium of cultured rat intestinal cells caused an increase in the % incorporation of radiolabel from [^{3}H]acetate into a polar lipid fraction with chromatographic properties similar to oxysterols. This increase was attenuated by agents that prevented the inhibition of reductase activity by LDL. One of these agents was ketoconazole, a known inhibitor of lanosterol 14α-demethylase and other cytochrome P450-linked reactions. Treatment of

Abbreviations: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; HPLC, high performance liquid chromatography; ACAT, acyl CoA:cholesterol acyl transferase; MVA, mevalonolactone; MC, miconazole; KC, ketoconazole; [^{3}H-CL]LDL, [^{3}H]cholesteryl linoleate-labeled LDL.

[1]A portion of this work was presented at the 72nd Annual Meeting of FASEB, Las Vegas, NV, 1-4 May 1988 (Gupta, A. K., R. C. Sexton, and H. Rudney. 1988. *FASEB J.* **2:** 581 (abstract).

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IEC-6 cells with ketoconazole was shown to cause: *1*) reductase activity to respond in a biphasic manner with increasing concentrations of the drug; *2*) an inhibition of incorporation of radioactive precursor into cholesterol with a concomitant increase in the accumulation of radiolabel into lanosterol and 24(S),25-epoxylanosterol; and *3*) prevention of LDL suppression of reductase activity without interfering with LDL processing. During our screening of additional cytochrome P450 inhibitors for their ability to prevent LDL action on reductase activity, we observed that miconazole, an imidazole compound structurally similar to ketoconazole and possessing many of the same inhibitory properties with respect to lanosterol demethylation and cytochrome P450-dependent reactions (12), was unable to prevent LDL suppression of reductase activity. We, therefore, examined in greater detail the effects of miconazole on sterol biosynthesis and LDL suppression of reductase activity in IEC-6 cells. Our present results clearly indicate that both ketoconazole and miconazole have similar effects on reductase activity and endogenous sterol synthesis. However, the drugs differed in their ability to prevent LDL action on reductase activity such that ketoconazole totally abolished LDL action on reductase activity, whereas miconazole was unable to prevent LDL action on reductase. This difference in the effect of each drug on LDL suppression of reductase was not unique to IEC-6 cells, but was observed in several cultured cell lines of different tissue origin. Collectively, the data suggest that ketoconazole prevents the suppressive action of LDL on reductase activity by acting at a specific locus that is unaffected by miconazole. Additionally, the data indicate that de novo synthesis of cholesterol or epoxycholesterol is not required for the suppression of reductase activity by LDL.

GENERAL EXPERIMENTAL PROCEDURES

Materials

(R,S)-3-[^{14}C]HMG-CoA (52-57 mCi/mmol), (R,S)-[5-^{3}H]mevalonolactone (24 Ci/mmol), [^{3}H]acetate (1.6 Ci/mmol), cholesteryl-[1,2,6,7-^{3}H(N)]linoleate (100 Ci/mmol) were obtained from New England Nuclear (Dupont Co., DE). 25-Hydroxycholesterol was purchased from Steraloid, Inc. Sodium [^{125}I]iodide (17 Ci/mg) was purchased from ICN Pharmaceuticals. 25-Hydroxylanosterol and 24(S), 25-epoxy [^{3}H]lanosterol were prepared as described previously (13). Cell culture supplies were obtained either from M. A. Biological Associates or Grand Island Biological Co. All other chemicals were obtained from local sources and were of the highest purity grade available.

Cell culture

Rat intestinal epithelial cells (IEC-6 cells) obtained from American Type Culture Collection (CRL-1592) were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum as described previously (5). Prior to the addition of drugs, LDL, or radiolabeled compounds, cells were incubated 24-48 h in DMEM medium supplemented with lipoprotein-deficient fetal calf serum (LPDS, 2 mg protein/ml) as described in appropriate figure and table legends. All experiments were conducted using cells in the logarithmic phase of growth such that cells numbers at harvest approximated 30-40% of saturation density (250-300 μg of protein/dish). Ketoconazole, miconazole, [^{3}H]acetate, mevalonolactone, 25-hydroxycholesterol, 25-hydroxylanosterol, and 24(S), 25-epoxy-[^{3}H]lanosterol were added to the culture medium as solutions in absolute ethanol such that the final concentrations of ethanol did not exceed 1% (v/v). Control cells received equivalent amounts of ethanol. None of the treatments had any significant effect on the growth of the cells during the time course of these studies.

Human hepatoblastoma cells (HepG2) obtained from American Type Culture Collection (HB-8065) were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) as described previously (14). For experiments, cells were seeded at 5 × 10^{4} cells into petri dishes (60 × 15 mm) in DMEM with 10% FCS. Prior to the addition of the drugs and LDL, cells were incubated for 24-48 h with DMEM medium supplemented with LPDS (4 mg protein/ml). Human skin fibroblasts obtained from Human Mutant Genetic Repository (GM-0043) were maintained on Eagle's minimal medium supplemented with 10% FCS as described earlier (15). For experiments, cells were seeded at 2 × 10^{5} cells into petri dishes (60 × 15 mm) in DMEM with 10% FCS. Prior to the addition of the drugs and LDL, cells were incubated for 24-48 h with DMEM medium supplemented with LPDS (4 mg protein/ml). Chinese hamster ovary cells wild type (CHO-K1) and 4α-methyl sterol oxidase mutant cells (CHO-215) were kindly supplied by Dr. T. Y. Chang, Dartmouth College, NH. Cells were maintained in F-12 medium supplemented with 10% FCS. For experiments, cells were seeded at 2 × 10^{5} cells into petri dishes (60 × 15 mm) in F-12 medium containing 10% FCS. Prior to the addition of the drugs and LDL, the cells were incubated for 24-48 h with F-12 medium supplemented with LPDS (4 mg protein/ml).

Lipoproteins

Human LDL (d $\leq$ 1.063 g/ml) was isolated by centrifugation of human serum after adjusting the density with

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KBr (16). Lipoprotein-deficient serum (d > 1.215 g/ml) was prepared from fetal calf serum by centrifugation after adjusting the density with KBr. The lipoprotein preparations were stored at 4°C after dialysis against 0.15 M NaCl containing 3 mM EDTA, pH 7.4. The concentration of LDL was expressed in terms of protein content (μg/ml). Preparation of ^{125}I-labeled LDL and its degradation by IEC-6 cells was studied as described previously (2, 5). ^{125}I radioactivity was determined in a gamma counter with 80% efficiency.

Measurement of HMG-CoA reductase activity

The activity of HMG-CoA reductase was determined in a detergent extract of monolayers as described before (5, 17). One unit of enzyme activity equals the formation of 1 pmol of mevalonate/min. The results are presented as the average of triplicate determinations (dishes) at each data point. The variation in data did not exceed 5% of the mean.

Separation of various nonsaponifiable lipids by HPLC

For measurement of nonsaponifiable lipids, the cells were incubated with [^{3}H]acetate as described in the legend of the figures and tables. At the end of incubation, medium was removed for extraction of total lipids into chloroform-methanol 1:2 (v/v) as described before (3). The monolayers were rinsed two times with 2 ml of ice-cold saline and total cellular lipids were extracted in hexane-isopropanol 60:40 (v/v) as described before (5, 17). In a separate experiment, the extraction efficiency of both [^{14}C]cholesterol and [^{3}H]25-hydroxycholesterol, as a representative polar sterol, was found to be greater than 95% using this lipid extraction solvent. After saponification of this extract, [^{3}H]25-hydroxycholesterol was extracted in hexane (more than 90%). Thus routinely, lipid extracts of cells and medium were combined and saponified prior to the extraction of free sterols in hexane (5, 14). The lipid components of the hexane extract were separated by high performance liquid chromatography (HPLC) on a 0.46 × 25 cm Zorbax ODS column (Dupont-New England Nuclear) as previously described (13, 17). Identity of sterol fractions was assigned by their elution in 100% methanol (1 ml/min) at the retention times of authentic standards: 25-hydroxycholesterol, 6.0 min; 24(S), 25-epoxycholesterol, 8.0 min; 24(S), 25-epoxylanosterol, 9.6 min; desmosterol, 19.3 min; lanosterol, 23.0 min; and cholesterol, 24.7 min (see Fig. 3).

Preparation and cellular metabolism of [^{3}H]cholesteryl linoleate-labeled LDL ([^{3}H-CL]LDL)

[^{3}H-CL]LDL was routinely prepared by a slight modification of the method of Brown, Dana, and Goldstein (18) as described previously (5). The sterilized [^{3}H-CL]LDL preparation contained between 10 and 25 μCi/mg of LDL protein and was stored at 4°C. For determination of degradation of [^{3}H-CL]LDL, the cells were labeled as described in the legend to the table. At the end of incubation, the monnolayers were rinsed two times with 2 ml of ice-cold saline. The total cellular lipids were extracted with 5 ml of hexane-isopropanol 60:40 (v/v). [^{14}C]Cholesteryl oleate (25 μg, 25,000 dpm), cholesteryl palmitate (25 μg), and cholesteryl linoleate (25 μg) were included in each extraction tube as a carrier to monitor the recovery. The total lipid extracts were spotted on 20 × 20 cm glass-backed silica gel 60 thin-layer chromatography plates (Merck 5763) impregnated with silver nitrate, prepared by immersion of the plates into a solution of 5% (w/v) silver nitrate in aqueous methanol (1:2, v/w). After development of the plates in benzene, the sterols were visualized by staining with 5% (v/v) anisaldehyde in sulfuric acid-ethyl alcohol 5:90 (v/v). The sterol fractions were then scraped into scintillation vials containing Scintiverse E and counted in a Beckman LS 3801 liquid scintillation counter.

Protein

Protein was estimated by the method of Schacterle and Pollack with a modification as described earlier (5). Bovine serum albumin was used as the standard.

RESULTS

Effect of ketoconazole and miconazole on HMG-CoA reductase activity and sterol synthesis in the presence and absence of LDL

The effects of ketoconazole and miconazole on the reductase activity of IEC-6 cells, incubated in the presence and absence of LDL, are shown in **Fig. 1**. In the absence of LDL, the reductase activity showed a biphasic response to increasing concentrations of ketoconazole (Fig. 1A) and miconazole (Fig. 1B). At low concentrations (< 2 μM) both drugs inhibited reductase activity by more than 50%. As the medium concentrations of each drug increased to 10 μM, the reductase activity was restored to control values, whereas, above 10 μM, both drugs significantly stimulated the reductase activity over the control values. In the case of ketoconazole at 30 μM, there was a 25% increase in reductase activity, whereas miconazole at a lower concentration of 20 μM elicited a 90% increase over control. In cells incubated with LDL (25 μg/ml), the reductase activity was inhibited by 80%. Addition of ketoconazole (> 10 μM) to the medium totally abolished the inhibition of reductase activity by LDL (Fig. 1A). On the other hand, miconazole at concentrations as high as 20 μM was unable to prevent the suppression of reductase activity by LDL. Above 20 μM, miconazole was toxic to the cells. This difference between ketoconazole and miconazole in the ability to prevent

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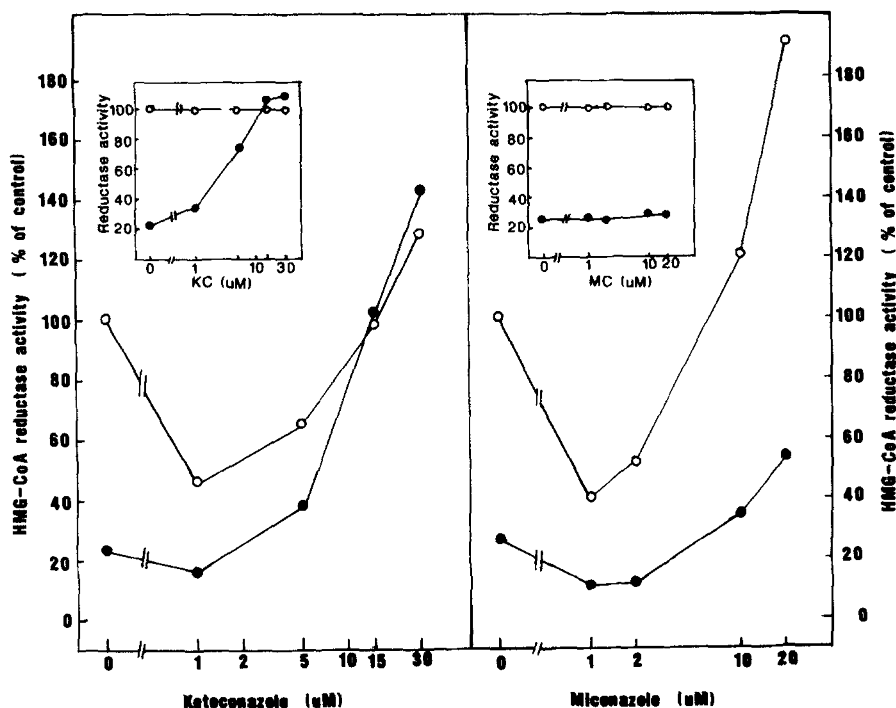

Fig. 1. Effect of varying concentration of ketoconazole (left) and miconazole (right) on reductase activity in the absence (○) and presence (●) of LDL. IEC-6 cells were seeded (day 0) at 2 × 10^5 cells/dish (60 × 15 mm) in 2 ml DMEM medium supplemented with 5% (v/v) fetal calf serum (FCS medium). The cells were refed 2 ml DMEM medium containing lipoprotein-deficient serum (LPDS medium; protein 2 mg/ml) on days 3 and 4. On day 4, ketoconazole or miconazole was added at the indicated concentration in 20 μl of ethanol. The control cells received equivalent amounts of ethanol. After 1 h of pretreatment with drugs, LDL was added to half of the dishes at a final concentration of 25 μg/ml. After an additional 6 h of incubation, cells were harvested for determination of reductase activity as described under Experimental Procedures. The values represent the mean of triplicate determinations and the SE for each data point is ≤5% of the mean. The small inserted figure represents the data in Fig. 1A and 1B recast so that reductase activity in the presence of the drug, but in the absence of LDL, is taken to represent 100% and is plotted on the y axis.

LDL suppression of reductase activity was more clearly seen when the reductase activities were assumed to be 100% at each drug concentration in the absence of LDL (insert to Figs. 1A and 1B).

The increase in reductase activity caused by the drugs was seen in all experiments; however, the percentage increase varied from 25 to 300%. This variation in the stimulation of reductase activity by the drugs may be a function of the stage of cell growth in the culture. As shown in **Fig. 2**, when the medium was seeded with low and high cell numbers, marked differences in the extent of stimulation of reductase activity by each drug was observed. Thus, ketoconazole was able to stimulate reductase activity regardless of whether the medium was seeded with a high or low cell number. Miconazole, on the other hand, stimulated reductase activity to a greater extent in cultures seeded with low cell number compared to cultures seeded with high cell number. Since high cell number seeding rapidly generates a confluent cell culture, whereas with low cell number seeding the cells are still in the logarithmic phase of growth, the variations observed in the extent of stimulation of reductase activity by the drugs may be related to the proportion of cells in each stage of growth.

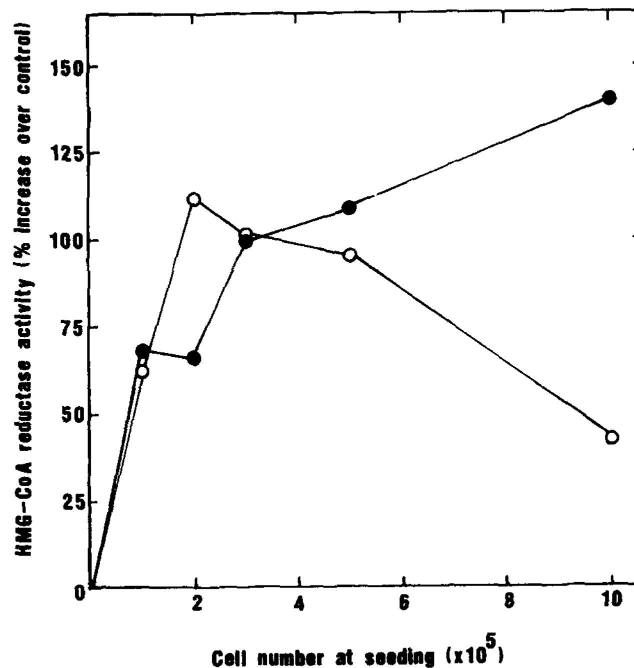

Fig. 2. Comparison of the stimulatory effect of ketoconazole (○) and miconazole (●) in IEC-6 cells seeded at different cell numbers. On day 0, the cells were seeded at the indicated number and grown for 3 days as described in the legend to Fig. 1. On day 4 cells were fed LPDS medium with either ethanol or ketoconazole (30 μM) or miconazole (10 μM). After 6 h of further incubation, cells were harvested for determination of reductase activity. The reductase activity (pmol/min per mg protein) in control cells was 211 ± 7 for 1 × 10^5 cells, 177 ± 3 for 2 × 10^5 cells, 150 ± 6 for 3 × 10^5 cells, 120 ± 9 for 5 × 10^5 cells and 124 ± 9 for 10 × 10^5 cells.

Since ketoconazole was known to inhibit several steps in the biosynthetic pathway of sterol and steroids (5, 12), it seemed reasonable to assume that the observed difference in LDL suppression of reductase activity in the presence of the drugs was due to each drug inhibiting sterol bio-

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synthesis at different sites. In **Fig. 3**, the effects of each drug on the incorporation of radioactivity from [^{3}H]acetate into several sterol intermediates of the cholesterol biosynthetic pathway are shown. In the absence of the drugs and LDL, the radiolabel from [^{3}H]acetate appeared in the cholesterol, desmosterol, and lanosterol fractions. Addition of either ketoconazole (30 μM) or miconazole (10 μM) to the medium caused a complete inhibition in the incorporation of radioactivity in the cholesterol and desmosterol fractions with a concomitant increase in the lanosterol fraction. Thus, both drugs appeared to inhibit the activity of lanosterol 14α-demethylase to the same extent. Addition of LDL did not cause any major change in the distribution of radioactivity either in the presence or absence of the drugs. The results outlined in Fig. 3 suggest that the different effects of ketoconazole and miconazole on LDL suppression of reductase activity are not related to differences in their ability to inhibit endogenous cholesterol synthesis at the lanosterol 14α-demethylation step.

Effect of ketoconazole and miconazole on the metabolism of intermediates of cholesterol biosynthesis

Earlier studies with rat intestinal epithelial cells (5) and Chinese hamster ovary cells (19) indicated that ketoconazole inhibited acetate incorporation into cholesterol and caused an accumulation of intermediates of cholesterol and epoxycholesterol biosynthesis. The accumulated intermediate(s) inhibited HMG-CoA reductase activity only after removal of ketoconazole from the medium, demonstrating that ketoconazole prevented their further metabolism to reductase suppressors. To check the possibility that miconazole differed from ketoconazole in its ability to prevent the metabolism of intermediate(s) to reductase suppressor(s), cells were treated for 24 h with ketoconazole (30 μM) or miconazole (10 μM), followed by refeeding the cells either fresh medium without drugs or medium with ketoconazole or miconazole. The results are presented in **Table 1**. In control cells (experiments A and B), the reductase activity was stimulated at 6 h after

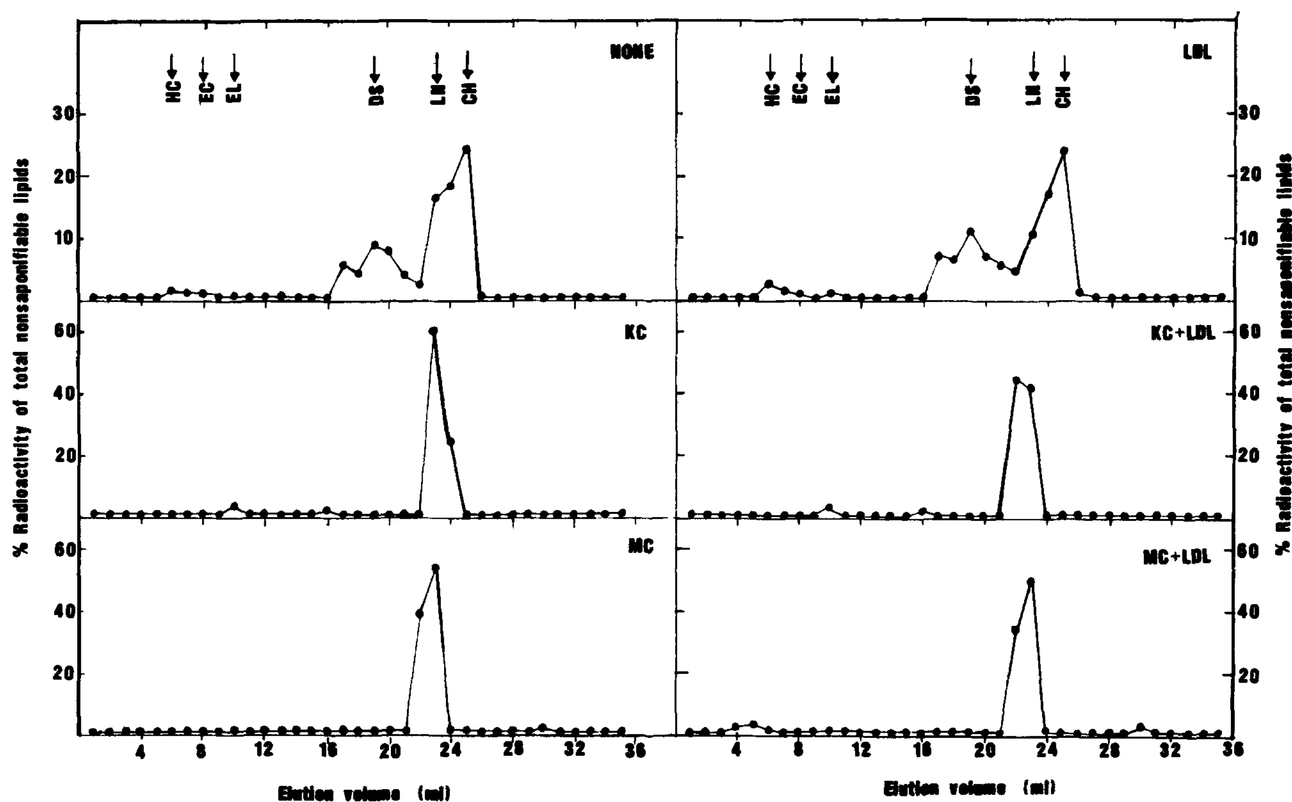

Fig. 3. Effect of ketoconazole and miconazole on [^{3}H]acetate incorporation in nonsaponifiable lipids in the absence and presence of LDL. IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed fresh LPDS medium containing ketoconazole (30 μM) or miconazole (10 μM) or ethanol. After 1 h of pretreatment with the drugs, LDL (25 μg/ml) was added to half of the dishes. Five h after the addition of LDL, the cells were pulsed with [^{3}H]acetate (10 μCi/dish) for 1 h. Triplicate dishes were harvested from each group to determine the incorporation of radiolabel into nonsaponifiable lipids by HPLC as described in the Experimental Procedures. Total dpm incorporated into nonsaponifiable lipids: in control, 0.979 $\times$ 10^5 for control; 0.989 $\times$ 10^5 for ketoconazole; 1.598 $\times$ 10^5 for miconazole; 0.479 $\times$ 10^5 for LDL; 1.248 $\times$ 10^5 for LDL and ketoconazole; 1.237 $\times$ 10^5 for LDL and miconazole. HC, hydroxycholesterol; EC, epoxycholesterol; EL, epoxylanosterol; DS, desmosterol; LN, lanosterol; CH, cholesterol.

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TABLE 1. Effect of the withdrawal of the drugs on HMG-CoA reductase activity in IEC-6 cells

Treatment Medium	HMG-CoA Reductase Activity			
	After 24 h Pretreatment	After 6 h Refeeding		
		Control	Miconazole	Ketoconazole
	pmol/min per protein			
Experiment A				
Control	78	82	92	206
Miconazole	202	53	223	222
Experiment B				
Control	113	173	305	285
Ketoconazole	222	134	219	243

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, the cells were fed LPDS medium containing either ketoconazole (30 μM) or miconazole (10 μM) or ethanol. After 24 h of incubation, triplicate dishes were harvested from each group. The rest of the dishes were fed LPDS medium containing either ketoconazole (30 μM) or miconazole (10 μM) or ethanol. The cells from triplicate dishes were harvested after 6 h for the determination of reductase activity. Experiments A and B represent experiments at different times.

refeeding the cells fresh medium containing either miconazole or ketoconazole compared to cells refed only fresh medium. The degree of stimulation noted for each drug, however, varied from that presented in Fig. 1 and may have been due to the stage of cell growth as noted in Fig. 2. In cells pretreated for 24 h with either miconazole or ketoconazole, the reductase activity was stimulated by both drugs, 78 to 202 by miconazole (Expt. A) and 113 to 222 by ketoconazole (Expt. B). Refeeding the drug-pretreated cells with fresh medium containing either drug resulted in little change in the reductase values. However, refeeding the drug-pretreated cells with fresh medium without added drugs resulted in a 40-73% inhibition of reductase activity, e.g., from 202 to 53 in Expt. A and 222 to 134 in Expt. B. These results support the hypothesis that reductase-suppressive intermediates accumulate during the pretreatment period of cells with either drug and that these intermediates are not further metabolized or allowed to act in the continued presence of either drug. Removal of the drugs allowed the expression of these reductase-suppressive intermediates.

In our previous study (5) on the effect of ketoconazole on sterol biosynthesis, we showed that epoxylanosterol accumulated in the presence of the drug, because the conversion of epoxylanosterol to epoxycholesterol was inhibited. The possibility that miconazole also inhibited the formation of epoxycholesterol was confirmed by following the metabolism of 24(S), 25-epoxy-[^{3}H]lanosterol in IEC-6 cells in the presence and absence of the drugs (**Table 2**). In the absence of either drug, 65% of 24(S), 25-epoxy-[^{3}H]lanosterol was metabolized to 24(S), 25-epoxy-[^{3}H]cholesterol. Addition of either drug to the medium caused an increase in the radiolabel associated with the cells; however, both drugs completely prevented the metabolism of 24(S), 25-epoxylanosterol to 24(S), 25-epoxycholesterol. These results and those of Fig. 3 and Table 1 indicate that both drugs inhibit to a similar extent the endogenous synthesis of cholesterol and the conversion of epoxylanosterol to a reductase suppressor, but differed in their ability to prevent the suppressive action of LDL on reductase activity.

Each drug was unable to prevent the inhibition of reductase activity by 25-hydroxycholesterol, 25-hydroxylanosterol (**Table 3**), or mevalonolactone (**Fig. 4**). These results suggested that since the drugs were unable to prevent the action of an inhibitory oxysterol once it was formed, the inhibitory action of LDL did not involve introduction into the cells of a preformed oxysterol with the same potency as 25-hydroxycholesterol or the formation of the putative inhibitory product of mevalonate metabolism. These observations do not rule out the possibility

TABLE 2. Prevention of 24(S),25-epoxy [^{3}H]lanosterol metabolism by ketoconazole and miconazole in IEC-6 cells

Cellular Uptake and Distribution of Radiolabel	[^{3}H]dpm per Dish		
	Control	+ Ketoconazole	+ Miconazole
Total uptake	20330	38469	37500
S-EC	13369	ND	ND
S-EL	6961	38469	37500

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, the cells were fed LPDS medium containing either ethanol or ketoconazole (30 μM) or miconazole (10 μM). After 1 h of pretreatment, 24(S),25-epoxy [^{3}H]lanosterol (70,000 dpm/dish) was added. At the end of 6 h, cellular lipids were extracted as described in Experimental Procedures for analysis by HPLC on a Zorbax ODS column in 100% methanol (1 ml/min). Under these conditions, the retention times for S-EC (24,25-epoxycholesterol) and S-EL (24,25-epoxylanosterol) were 7.9 and 9.5 min, respectively; ND, nondetectable.

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TABLE 3. Effect of ketoconazole and miconazole on suppression of reductase activity by 25-hydroxycholesterol and 25-hydroxylanosterol in IEC-6 cells

Sterol Added	Conc.	HMG-CoA Reductase Activity		
		Control	Ketoconazole (30 μM)	Miconazole (10 μM)
	ng/mg		*pmol/min per mg protein*	
25-Hydroxylanosterol	0	71 ± 3	157 ± 10	90 ± 6
25-Hydroxylanosterol	25	58 ± 3	97 ± 11	70 ± 7
25-Hydroxylanosterol	250	12 ± 1	34 ± 4	19 ± 2
25-Hydroxycholesterol	0	69 ± 2	170 ± 3	109 ± 8
25-Hydroxycholesterol	25	57 ± 2	90 ± 8	93 ± 4
25-Hydroxycholesterol	250	8 ± 1	21 ± 2	24 ± 1

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, the cells were fed LPDS medium containing either ethanol or ketoconazole (30 μM) or miconazole (10 μM). After 1 h of pretreatment with the drugs, indicated concentrations of 25-hydroxylanosterol or 25-hydroxycholesterol were added. After an additional 6 h, triplicate dishes were harvested for the determination of reductase activity.

that a regulatory molecule with lesser potency than 25-OH cholesterol may be transported on LDL (see beyond).

Processing and metabolism of low density lipoprotein in the presence of miconazole and ketoconazole

We have previously shown that although ketoconazole did increase the overall uptake of LDL it had no major effect on the intracellular processing of LDL in IEC-6 cells (5). Cholesterol esterification was also inhibited by ketoconazole; however, this inhibition has been shown to be unrelated to the prevention of the suppression of reductase activity by LDL (5). To determine whether the effects of miconazole on LDL processing were similar to those of ketoconazole, we compared the effect of both drugs on hydrolysis of [^{3}H]cholesteryl linoleate-labeled LDL and re-esterification of free [^{3}H]cholesterol. Cells were incubated with [^{3}H]cholesteryl linoleate-labeled LDL in the presence and absence of the drugs for 6 h or 24 h and the results are presented in **Table 4**. The results show that each drug prevented the esterification of cholesterol measured as incorporation of [^{3}H]cholesterol into cholesteryl oleate and palmitate. Of the radioactivity associated with control cells in 6 h, 66% was accounted for as free cholesterol. Miconazole treatment alone had little effect on the release of free cholesterol compared to control (156,521 dpm in control to 141,341 dpm in miconazole-treated cells), whereas ketoconazole treatment caused a 25% inhibition in the release of free cholesterol compared to control cells (156,521 dpm in control to 118,544 dpm in ketoconazole-treated cells). This decrease in the release of free cholesterol by ketoconazole during the 6-h treatment period could not account for the complete prevention of LDL suppression of reductase activity. In cells incubated with ketoconazole and [^{3}H-CL]LDL for 24 h there was a significant increase (35%) in the release of free cholesterol compared to control (220,789 dpm in control incubations to 299,950 dpm in ketoconazole incubations) without suppression of reductase activity by LDL. Ketoconazole and miconazole treatment caused an increase in the amount of [^{3}H]cholesteryl linoleate-labeled LDL radioactivity associated with the cells during both 6-h and 24-h periods. The increase in [^{3}H]cholesteryl linoleate-labeled LDL associated with the cells was further evaluated by following the binding, uptake, and a degradation of ^{125}I-labeled LDL in the pres-

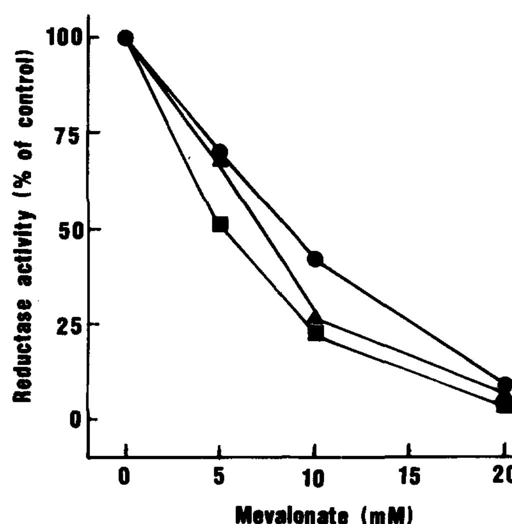

Fig. 4. Effect of drugs on mevalonolactone inhibition of HMG-CoA reductase in IEC-6 cells. IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed LPDS medium containing either ethanol (●) or 30 μM ketoconazole (■) or 10 μM miconazole (▲). After 1 h pretreatment with the drugs, indicated concentrations of mevalonolactone were added. After an additional 6 h of incubation, cells were harvested for determination of reductase activity. The values represent the mean of triplicate determinations and SE for each data point is 5% of the mean. The control reductase (pmol/min per mg protein) activity values were 115 ± 2, 329 ± 12, and 228 ± 3 for ethanol, ketoconazole, and miconazole, respectively.

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TABLE 4. Metabolism of [^{3}H-Cl]LDL in IEC-6 cells grown in the presence and absence of the drugs

	Distribution of [^{3}H]cholesterol from [^{3}H-Cl]LDL					
	Experiment A			Experiment B		
Sterol Fractions	Control	Miconazole (10 μM)	Ketoconazole (30 μM)	Control	Miconazole (10 μM)	Ketoconazole (30 μM)
	dpm × 10^{-2}/mg protein					
Free cholesterol	1565 ± 125	1413 ± 97	1185 ± 49	2208 ± 55	ND	3000 ± 40
Cholesteryl oleate	169 ± 12	48 ± 5	57 ± 6	334 ± 17	ND	68 ± 16
Cholesteryl palmitate	116 ± 7	15 ± 1	11 ± 2	196 ± 9	ND	9 ± 2
Cholesteryl linoleate	539 ± 50	1499 ± 166	1908 ± 166	363 ± 66	ND	4540 ± 577
Total dpm associated with cells	2390 ± 194	2975 ± 269	3162 ± 17	3101 ± 146		7616 ± 644

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, the cells were fed fresh LPDS medium containing either ethanol or ketoconazole (30 μM) or miconazole (10 μM). After 1 h of pretreatment with the drugs, [^{3}H]cholesteryl linoleate-LDL (20 μg/ml; 37.9 × 10^{6} dpm/mg of LDL protein) was added.to the medium. After 6 h incubation ((Expt. A) or after 24 h incubation (Expt. B), the cellular lipids were extracted for determination cf free and esterified cholesterol; ND, not determined.

ence and absence of the drugs. As shown in **Table 5**, incubation of cells with either ketoconazole or miconazole for 24 h resulted in an increase in ^{125}I-labeled LDL binding and total cell-associated radioactivity compared to untreated cells. The net amount of degradation of the LDL apoprotein was also higher in the drug-treated cells compared to controls. However, unhydrolyzed LDL measured as cell-associated and receptor-bound was significantly higher in drug-treated cells. These results provide a basis for the observations listed in Tables 4 and 5 with respect to the increased 24-h uptake of labeled LDL and the increase in the release of free cholesterol by the drugs. Despite the fact that both drugs induced similar increases in the uptake, binding, and degradation of LDL and the release of free cholesterol, miconazole was unable to prevent the down-regulation of reductase activity, whereas ketoconazole effectively prevented LDL action on reductase activity. The re-esterification of [^{3}H]cholesterol released from LDL was greatly inhibited by both drugs, indicating an inhibition of ACAT activity. These and earlier results (5) clearly indicate that ACAT activity is not involved in the prevention of the LDL effect on reductase activity. Collectively, these results suggest that the ability of ketoconazole to prevent LDL suppression of reductase is due to an inhibition of a site in the LDL pathway that is not sensitive to miconazole.

Effect of ketoconazole and miconazole on modulation of LDL action in different cell lines

In order to determine whether the difference in the effects of ketoconazole and miconazole on LDL suppression of reductase activity was unique to IEC-6 cells, we examined, in the absence of LDL, the effects of each drug on the reductase activity in cells of varied origin, e.g., human skin fibroblasts (GM-43), transformed human liver cells (HepG2), wild type Chinese hamster ovary cells (CHO-K1), and a 4α-methyl sterol oxidase mutant of Chinese hamster ovary cells (CHO-215). Previously Favata et al. (19) have shown that in CHO-K1 cells increasing concentration of ketoconazole and miconazole caused a biphasic response in reductase activity, where-

TABLE 5. Effect of miconazole and ketoconazole on binding, uptake, and degradation of ^{125}I-labeled LDL and LDL suppression of reductase activity in IEC-6 cells

	^{125}I-Labeled LDL			HMG-CoA Reductase Activity	
Drugs	Receptor-Bound	Cell-Associated	Cell-Degraded	– LDL	+ LDL
	ng/mg cell protein			*pmol/min per mg protein*	
None	65 ± 17	721 ± 114	2444 ± 83	147 ± 8	20 ± 3
Ketoconazole (30 μM)	150 ± 26	1445 ± 21	2988 ± 17	456 ± 29	522 ± 15
Miconazole (10 μM)	121 ± 6	1262 ± 140	3424 ± 146	243 ± 12	59 ± 3

IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, the cells were fed LPDS with medium containing either ethanol or ketoconazole (30 μM) or miconazole (10 μM). After 1 h pretreatment, ^{125}I-labeled LDL was added (25 μg/ml, 116 cpm per ng protein). Triplicate dishes were harvested after 24 h for determination of reductase activity and ^{125}I-labeled LDL processing. Briefly, after incubation, medium was collected for determination of cell-degraded LDL. To determine the receptor-bound LDL, cells were treated with buffer containing heparin (0.4% w/v) for 1 h at 4°C. The buffer was carefully removed and assayed for radioactivity. Cell-associated (internalized plus undegraded) LDL was determined after digesting the cells in 0.1 N NaOH for 24 h.

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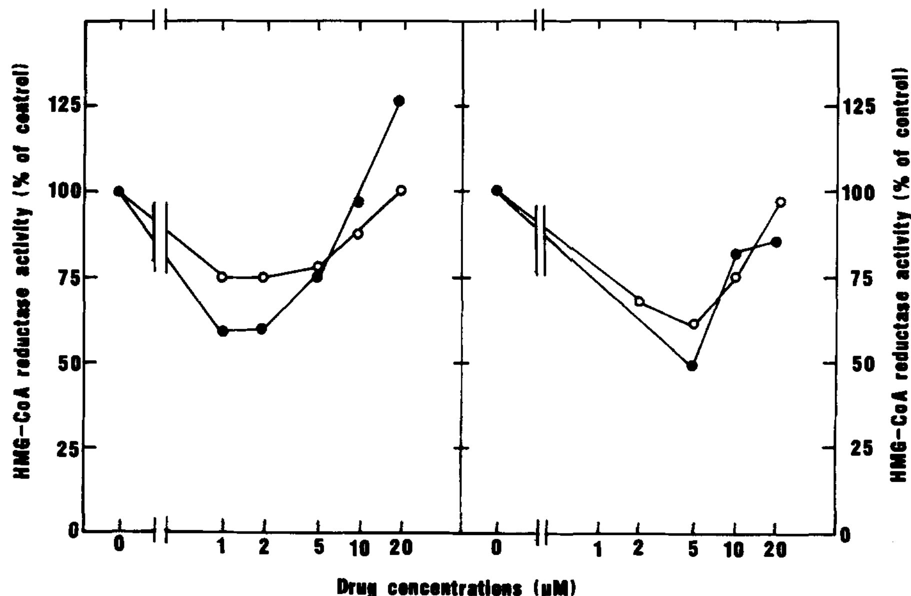

Fig. 5. The effect of ketoconazole and miconazole on reductase activity in HepG2 cells (left) and GM-43 cells (right). HepG2 cells (5 × 10^4 cells/dish) and GM-43 cells (2 × 10^5 cells/dish) were seeded on day 0 in DMEM medium supplemented with 10% FCS. On days 3 and 4, the cells were fed fresh DMEM medium containing LPDS (4 mg/ml). On day 4, cells were fed medium containing either ethanol or indicated concentrations of ketoconazole (●) or miconazole (○). Cells were harvested for determination of reductase activity after 6 h of the incubation as described in Experimental Procedures.

as in the CHO-AR-45 mutant no biphasic reductase response was observed (20). In GM-43 cells and HepG2 cells we have found, as in IEC-6 cells, that the reductase activity varied in a biphasic manner in response to increasing concentrations of each drug (**Fig. 5**), therefore, in wild type cells the reductase responds in a biphasic manner to increasing drug concentrations. In order to examine whether the drugs had a differential effect on LDL suppression of reductase activity, a concentration of drug was chosen such that the reductase activity was restored to near the control value. In the lanosterol demethylation mutant, CHO-215, the drug concentrations were the same as for the wild type, CHO-K1 cells, since in lanosterol demethylation mutants no biphasic response is observed (20). The results presented in **Table 6** show that treatment of all cell lines with LDL caused an inhibition of reductase activity. Ketoconazole prevented the inhibition of reductase activity by LDL in all cell lines tested,

TABLE 6. Modulation of LDL suppression of reductase activity by ketoconazole and not by miconazole in several cell lines

Cell Lines	Additions	HMG-CoA Reductase Activity		
		Control	Ketoconazole (30 μM)	Miconazole (10 μM)
			pmol/min per mg protein	
GM-43	− LDL	193 ± 20	144 ± 6	144 ± 4
GM-43	+ LDL (25 μg/ml)	15 ± 5	114 ± 22	20 ± 2
HepG2	− LDL	1014 ± 28	1713 ± 104	1066 ± 40
HepG2	+ LDL (100 μg/ml)	461 ± 21	1994 ± 15	439 ± 17
CHO-K1	− LDL	289 ± 13	320 ± 8	317 ± 15
CHO-K1	+ LDL (25 μg/ml)	81 ± 6	358 ± 14	91 ± 15
CHO-215	− LDL	314 ± 4	355 ± 13	319 ± 8
CHO-215	+ LDL (25 μg/ml)	135 ± 9	386 ± 25	129 ± 2

The cells from four cell lines were grown for 3 days as described in Experimental Procedures. On day 4, the cells were fed LPDS medium containing either ethanol or ketoconazole or miconazole. After 1 h pretreatment with the drugs, LDL was added. After an additional 6 h, triplicate dishes from each group were harvested for determination of reductase activity.

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whereas miconazole was unable to prevent LDL inhibition of reductase activity in all cell lines. These results clearly show that in a variety of cell lines of different tissue origin ketoconazole and miconazole have different effects on the suppression reductase activity by LDL and, therefore, the difference in the effects of each drug on LDL suppression of reductase activity in IEC-6 cells is not unique to this line.

DISCUSSION

We have previously shown that treatment of cultured cells with inhibitors of cholesterol biosynthesis and cytochrome P450-linked reactions prevents the LDL suppression of reductase activity without affecting the processing of LDL (9). These observations led to an hypothesis that a cytochrome P450-linked reaction was involved in the regulation of reductase activity by LDL (5, 9). Such a reaction would generate an oxysterol via the enzymic oxidation of endogenous cholesterol, or exogenous cholesterol entering the cell via the LDL pathway, or from intermediates of cholesterol or epoxycholesterol biosynthesis (5). In the present investigation, we have examined in greater detail these possibilities with agents that exert a differential effect on LDL regulation of reductase activity. Earlier we noted that miconazole (21) and ketoconazole (12, 22), drugs with very similar inhibitory effects on cytochrome P450-linked reactions, have different effects on the suppression of reductase activity by LDL (23). A comparison of the effects of each drug on the incorporation of [^{3}H]acetate into sterol intermediates suggested that the suppression of reductase activity by LDL was not mediated by generation of a suppressor from endogenous intermediates of either the cholesterol or epoxycholesterol pathway. These experiments, however, left open the possibility that a specific cytochrome P450-linked reaction, inhibited by ketoconazole and not by miconazole, could generate regulators of HMG-CoA reductase activity by acting on cellular cholesterol, endogenously or exogenously supplied, or on molecules supplied from another source. Support for this hypothesis is based on the following analysis of our current data.

In IEC-6 cells the reductase activity responded in a biphasic manner to both miconazole and ketoconazole, i.e., inhibition at low drug concentrations (<5 μM) and either no inhibition or to some degree a stimulation of activity as either drug concentration increased (>10 μM) (Fig. 1). The stimulation of reductase activity by high concentrations of miconazole appeared to be dependent on cell density and cell growth. Ketoconazole, on the other hand, stimulated reductase activity independent of cell growth. We have previously explained the biphasic response of reductase activity to ketoconazole on the basis that low concentrations of the drug caused a partial inhibition of lanosterol demethylation resulting in an accumulation of reductase-suppressive polar sterols (5). As the concentration of the drug was increased, the inhibition of polar sterol formation was complete and the inhibition of reductase was prevented. In our current experiments, we observed that an accumulation of lanosterol occurred during a period of pretreatment with miconazole or ketoconazole (Fig. 3). When pretreatment was followed by refeeding the cells LPDS medium without drugs, inhibition of reductase activity occurred which may be accounted for by an increase in the formation of polar sterols from accumulated lanosterol (Table 1). However, when miconazole or ketoconazole was present in the refeeding medium, the reductase activity was not suppressed and no metabolism of accumulated lanosterol to suppressor(s) was observed. These observations related to the biphasic response of reductase activity to both drugs have been verified by Favata et al. (19) using Chinese hamster ovary cells and Trzaskos et al. (24) in rat liver microsomes. Additional studies by Favata et al. (19) showed that in a mutant cell line (AR-45) which lacks lanosterol 14α-demethylase activity both ketoconazole and miconazole failed to elicit a biphasic response in reductase activity. Both our work and that of Favata et al. (19) support the concept that the biphasic response of reductase activity to both drugs is related to the ability of cells to metabolize lanosterol derivatives to suppressor products. Kempen et al. (25), however, did not observe the biphasic response with ketoconazole in a HepG2 cell line as we have reported (Fig. 5). We are unable to account for the different results obtained by the two laboratories other than to suggest that the difference in growth conditions may play a role. The cell protein to drug concentration ratio (mg/μM) in the studies reported by Kempen et al. (25) was 4–5 times greater than reported in this work. There is also the possibility of variation in the HepG2 cell lines used.

In primary hepatocyte cultures (20) and rat liver microsomes (24) Trzaskos et al. have clearly shown that a lanosterol metabolite, 3β-hydroxylanost-8-en-32-aldehyde, which accumulates in the presence of low concentrations of miconazole and ketoconazole, may be the putative regulatory oxysterol responsible for the nadir of the biphasic curve of reductase activity. At high concentrations of either drug, the formation of the 32-aldehyde metabolite of lanosterol decreased and reductase activity increased towards control values. In the present studies, we have used concentrations of miconazole (10 μM) and ketoconazole (30 μM) that completely inhibit the demethylation of either lanosterol (Fig. 3) or epoxylanosterol (Table 2), thus preventing formation of lanosterol 32-aldehyde and the subsequent inhibition of reductase activity. Under these conditions, LDL suppression of reductase activity was prevented by ketoconazole,

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but not by miconazole in IEC-6 cells (Fig. 1) or several cell lines of different tissue origin (Table 6). These results indicated that sterol synthesis beyond the lanosterol and epoxylanosterol demethylation step was not required for LDL to suppress reductase activity.

Evidence by Burki, Logel, and Sinensky (26), using a squalene cyclase inhibitor, TMD, to inhibit sterol synthesis in CHO cells but not LDL suppression of reductase activity, stongly suggests that sterol synthesis beyond the squalene cyclase step is not required for LDL action on reductase activity. Therefore, our observation that miconazole and ketoconazole inhibit sterol synthesis at a post-squalene cyclase step, i.e., a cytochrome P450-dependent sterol demethylation step, does not account for the differential effects of miconazole and ketoconazole to prevent LDL suppression reductase activity. The possibility exists that an alternate cytochrome P450-dependent process with differing sensitivity to the drugs may be responsible for producing the LDL effect on reductase activity.

Trzaskos et al. (20) and Favata et al. (19), however, did observe a major difference in the effect of ketoconazole and miconazole on the accumulation of epoxylanosterol and squalene diepoxide in CHO-K1 wild type cells and the mutant CHO cell line AR-45. In each cell type they observed an accumulation of epoxylanosterol in the presence of ketoconazole, but no accumulation in the presence of miconazole. In IEC-6 cells, we have not observed an effect of either drug on squalene diepoxide formation, but we have observed an increase in the accumulation of epoxylanosterol (4- to 5-fold) in the presence of ketoconazole relative to that obtained in the presence of miconazole when cells were pulsed for >6 h (data not shown). In a separate experiment, similar to that reported in Table 1, the intracellular level of epoxylanosterol was raised by incubation of cells in the presence of ketoconazole for 24 h. Refeeding the cells fresh medium containing either ketoconazole or miconazole, at concentrations known to prevent sterol demethylation, still allowed the down-regulation of reductase activity by LDL in cells refed miconazole, but not ketoconazole. The same result was obtained when epoxylanosterol was added to the medium of IEC-6 cell cultures that were incubated with miconazole and LDL. No influence of epoxylanosterol on the down-regulation of reductase by LDL in the presence of miconazole could be observed (data not shown). In a different cultured cell line, HepG2, both miconazole and ketoconazole inhibited completely the demethylation of lanosterol. In this cell line, squalene diepoxide rather than epoxylanosterol accumulates under ketoconazole treatment, whereas neither accumulate under miconazole treatment. Under these conditions, LDL still suppressed reductase activity in the presence of miconazole but not in cells treated with ketoconazole (data not shown). Further work is required to establish the cause of the difference between the drugs with respect to the accumulation of epoxylanosterol and squalene diepoxide in different cell lines, but at this time we have no evidence to relate their presence or absence in cells to the mechanism by which ketoconazole and miconazole differ in their ability to prevent LDL suppression of reductase activity.

In an earlier report we have shown that ketoconazole was an ACAT inhibitor in IEC-6 cells (5). In agreement with that work, Table 4 shows that the release of free cholesterol from cholesteryl linoleate in LDL is relatively unaffected by both ketoconazole and miconazole, but the re-esterification via ACAT activity is inhibited. Thus, miconazole resemble ketoconazole with respect to its inhibitory action on ACAT. We have also reported (5) that the inhibition of ACAT by ketoconazole was not related to its prevention of LDL suppression of reductase activity since a compound (Sandoz-58-035), which is a potent inhibitor of ACAT activity (27), was unable to prevent the down-regulation of reductase by LDL (5). In the present study, the general effects of miconazole resembled more closely those of ketoconazole than those of Sandoz-58-035, yet, despite being an ACAT inhibitor, miconazole was unable to prevent LDL inhibition of reductase. These results reinforce our earlier conclusion that ACAT activity is unrelated to the LDL down-regulation of reductase activity by LDL.

In a previous study we reported that ketoconazole increased the uptake of LDL by cells (5). In the current work, we have extended this observation and found that both ketoconazole and miconazole cause a similar increase in LDL uptake by cells. Further evaluation of this effect indicated that both drugs stimulated by twofold the binding and internalization of LDL (Table 5). The degradation of LDL was also increased due to drug treatment. A similar observation with respect to ketoconazole has been reported by Kempen et al. (25). These data suggest that both drugs may also interrupt in a similar manner the signalling system by which an increase in LDL uptake results in a down-regulation of the number of LDL receptors. Despite this additional similarity, the down-regulation of reductase activity is prevented by ketoconazole, but not by miconazole.

The suppression of reductase activity by a preformed oxysterol present on LDL seems unlikely in view of the observation that both ketoconazole and miconazole are unable to prevent the suppression of reductase activity by 25-hydroxycholesterol or 25-hydroxylanosterol (Table 4). Additionally, both drugs were unable to prevent mevalonolactone suppression of reductase activity suggesting that LDL does not inhibit reductase activity via generation of nonsterol or sterol products of mevalonate metabolism (28–32). On the other hand, these results do not rule out the possibility that a regulatory molecule with lesser potency than 25-hydroxycholesterol may be carried

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on LDL, and that the inhibitory effect of such a molecule might be more easily prevented by ketoconazole than miconazole.

Further support of the hypothesis that a cytochrome P450-linked reaction is involved in the mechanism by which LDL suppresses reductase activity is our observation that a series of cytochrome P450 inhibitors have been shown to prevent the down-regulation of reductase activity by LDL (9). Additionally, we have obtained preliminary evidence that in IEC-6 cells, LDL increases the formation of polar sterols that are inhibitory to HMG-CoA reductase activity (11). However, we have been unable to obtain this result consistently under our conditions of tissue culture. The possibility that LDL may induce the formation of a polar sterol in small amounts that may be acting at some regulatory locus remains open. In support of a regulatory role for oxysterols, observations have recently been reported demonstrating increases in hepatic polar sterol levels after cholesterol feeding in chicks by Aquilera et al. (33) and Calle and Gibbons in rats (34). Saucier et al. (35) have isolated and identified the 24- 25- and 26-hydroxycholesterol derivatives from heptocytes of rats fed a cholesterol diet.

In addition to possible involvement of a cytochrome P450-linked reaction that is inhibited by ketoconazole and not by miconazole, another explanation for the difference observed between the drugs with respect to LDL action on reductase activity may lie in the effect of each drug on lipid organization. Van den Bossche et al. (36) have explored the interaction of these drugs with lipids in multilamellar vesicles and have concluded that miconazole changes the organization of lipids in the vesicles without binding to the lipids, whereas, ketoconazole localizes in the multilayers of the vesicles without directly affecting lipid organization. Kempen et al. (25) raised the possibility that the effect of ketoconazole on LDL binding and uptake might be due to conformational changes in the LDL receptor induced by the drug. As we have demonstrated in this work, both ketoconazole and miconazole induce similar increases in LDL binding and uptake, but their effects on the down-regulation of reductase activity are dissimilar. Thus, it would appear that possible lipid interactions of the drugs with cellular membranes involved in LDL uptake, binding, and degradation might have a lesser effect compared to other factors. Several groups (37–40) have studied the role of structurally different imidazole antimycotic agents, including ketoconazole and miconazole, on hepatic cytochrome P450 hydroxylase reactions involved in testosterone metabolism. Varying effects of drugs on different steroid hydroxylases were observed resulting in selective inhibition of some monooxygenase reactions. Thus, the relative roles of membrane lipid interaction and specific structural properties of the drugs in the differential response of reductase activity to LDL are unknown at the present time. The studies reported here, showing the different effects of ketoconazole and miconazole on LDL suppression of reductase activity, despite their very similar biological actions in other systems, suggest that ketoconazole is acting at a specific step that may be a specific cytochrome P450-dependent reaction that is unaffected by miconazole. Additional studies on the effects of these drugs should provide more precise analysis of the mechanism of LDL suppression of reductase activity. ■

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