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Inhibition of Cholesteryl Ester Formation in Macrophages by Azole Antimycotics

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ABSTRACT. Cultured macrophages take up and metabolize cholesterol-containing liposomes, resulting in massive accumulation of cholesteryl esters in the cells. Using this system, the effects of azole antimycotics on cholesteryl ester formation were studied. Incubation of mouse peritoneal macrophages with ketoconazole, miconazole, or econazole (0.1–10 μM) resulted in concentration-dependent inhibition of cholesteryl ester synthesis from endocytosed cholesterol. IC_{50} values (concentration resulting in 50% inhibition) were 1.4 ± 0.1 μM, 4.1 ± 0.2 μM, and 3.6 ± 0.2 μM for ketoconazole, miconazole, and econazole, respectively. Complete inhibition was observed with 10 μM ketoconazole, and miconazole and econazole, each at 10 μM, caused 70 and 75% inhibition, respectively, of cholesteryl ester synthesis. The mechanism underlying the inhibition by ketoconazole was further studied. Ketoconazole did not appreciably block the uptake of liposomes or formation of triacylglycerol up to 10 μM. Interestingly, ketoconazole suppressed only 30% of 25-hydroxycholesterol-induced endogenous cholesterol esterification under conditions where esterification of endocytosed cholesterol was completely inhibited. Cytochemical studies with filipin-cholesterol staining revealed that ketoconazole induced massive accumulation of endocytosed cholesterol in macrophage phagolysosomes. These results indicate that ketoconazole inhibits cholesteryl ester formation in macrophages by blocking the intracellular transport of endocytosed cholesterol from lysosomes to the endoplasmic reticulum. BIOCHEM PHARMACOL **58**;3:447–453, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cholesterol; azole antimycotics; atherosclerosis; macrophages; lysosomes; intracellular transport

The early fatty streak lesions of atherosclerotic plaque are characterized by the presence of cholesteryl ester-laden foam cells [1]. Both electron-microscopic and immunological studies of these cells in such early lesions indicate that they are derived largely from circulating monocytes that have entered the subendothelial space [2]. Although the mechanism responsible for the conversion of subendothelial monocytes to foam cells is not fully understood, one process that has been proposed to contribute to the accumulation of cellular cholesteryl ester in these cells is the scavenger receptor pathway [3]. This pathway can mediate the recognition and catabolism of modified forms of lipoprotein that accumulate in the subendothelial space [4]. Modified lipoprotein, such as oxidatively modified LDL§, binds to scavenger receptors on the surface of macrophages and is delivered to lysosomes [5]. Within the lysosomes, the cholesteryl esters of the lipoprotein are hydrolyzed by lysosomal acid cholesterol esterase, liberating free cholesterol. The free cholesterol is transported to the endoplasmic reticulum, where much of it is re-esterified by ACAT. The resulting cholesteryl esters accumulate as cytoplasmic lipid droplets. Unlike the LDL receptor, the scavenger receptor is not down-regulated in response to an increase in the cellular cholesterol level [6]. This allows the cell to continue processing modified lipoproteins, despite increased levels of cellular cholesterol, and can lead to the accumulation of cholesteryl esters and generation of foam cells.

Agents that inhibit cholesteryl ester accumulation in macrophages are useful for elucidating the mechanism of foam cell formation, and also for the development of pharmacological drugs to prevent the formation of atherosclerotic plaques. A series of antimycotics have been reported to exert substantial effects on cellular cholesterol metabolism, including inhibition of cholesterol biosynthesis by liver cells [7, 8], cholesterol esterification by ACAT *in vitro* [9], and synthesis of steroid hormone by adrenal cells [10, 11].

In the present study, we examined whether azole antimycotics have any effect on cholesteryl ester formation from endocytosed cholesterol by cultured macrophages.

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§ *Abbreviations:* LDL, low-density lipoprotein; ACAT, acyl CoA:cholesterol acyltransferase; HBSS, Hanks' balanced salt solution; FITC, fluorescein-isothiocyanate; and HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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MATERIALS AND METHODS

Chemicals

The media used for the culture of mouse peritoneal macrophages were purchased as described previously [12]. 1, 2-Di[1-^{14}C]palmitoyl-glycerophosphocholine (100–200 mCi/mmol) and [4-^{14}C]cholesterol (50–60 mCi/mmol) were purchased from Dupont New England Nuclear. [9,10(n)-^{3}H]Oleic acid (5 Ci/mmol) was purchased from Amersham. Cholesterol, 25-hydroxycholesterol dicetylphosphate, pregnenolone, ketoconazole, miconazole, econazole, and FITC-dextran 70S were purchased from Sigma Chemical Co. Phosphatidylcholine and phosphatidylserine were purified by column chromatography from egg yolk and bovine brain, respectively. Filipin was obtained from Polysciences, Inc.

Cells

Mouse peritoneal macrophages were prepared as described previously [12]. Briefly, peritoneal cells were harvested from unstimulated female ICR mice using HBSS, then suspended at 2×10^6 cells/mL. Aliquots (1.0 mL) were dispensed into 12-well plastic microplates and incubated in a humidified CO_2 (5%) atmosphere at 37°. After 2 hr, each plate was washed three times with 1.0 mL of HBSS to remove unattached cells. The medium was immediately replaced with 1.0 mL of Dulbecco's modified Eagle's medium containing 6% lipoprotein-deficient serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) (hereafter referred to as medium A). Lipoprotein-deficient serum was prepared from fetal bovine serum according to the standard procedure [12].

Preparation of Liposomes

Multi lamellar liposomes were prepared as described previously [12]. Briefly, a mixture of lipids, typically phosphatidylcholine (1 μmol), phosphatidylserine (1 μmol), cholesterol (1.5 μmol), dicetylphosphate (0.2 μmol) and a trace amount of radioactive lipids as indicated, was dried in a rotary evaporator under reduced pressure. The dried lipids were dispersed with a vortex mixter in 1 mL of 0.3 M glucose solution at room temperature.

Preparation of BSA-[^{3}H]-Oleate Complex Solution

One hundred μCi of radiolabeled oleic acid which was evaporated to dryness was mixed with 2 mL of 0.25% BSA solution in medium A [12]. The mixture was filtered through a 0.22-μm-pore filter.

Metabolism of Liposomes by Cultured Macrophages

Mouse peritoneal macrophages attached to a plastic microplate after 2 hr of culture were washed with HBSS and replaced in 1 mL of medium A. After 2 hr of preincubation, 50 μL of liposomes containing radioactive lipids were added to each culture. After incubation for the indicated time, each plate was washed three times with HBSS, and the cells were lysed by adding 0.5 mL of 10 mM Tris–HCl (pH 7.4) containing 0.1% sodium dodecyl sulfate. An aliquot was taken for the determination of cellular protein and the radioactivity associated with the cells. Total lipids in the rest of the fraction were extracted by the method of Bligh and Dyer [13]. The lipids were then subjected to thin layer chromatography on a Silica Gel G plate using a mobile phase of hexane/ethyl ether/acetic acid (70:30:1 by volume). The spots corresponding to cholesteryl ester were scraped into a liquid scintillation vial in order to count their radioactivity.

25-Hydroxycholesterol-Stimulated Esterification of Endogenous Cellular Cholesterol

Cells were prepared as described above. After 2 hr of preincubation, 50 μL of BSA–[^{3}H]oleate complex solution and 5 μL of 25-hydroxycholesterol (500 μg/mL in ethanol) were added to each culture. After 6 hr of incubation, the cells were harvested and the radioactivity of the cellular cholesteryl ester fraction was counted as described above.

Staining of Cellular Unesterified Cholesterol with Filipin

Macrophage monolayers grown in microscope slide chambers were rinsed three times with 1 mL of HBSS and fixed with 1 mL of 3–4% phosphate-buffered (pH 7.4) formalin at room temperature for 10 min. Monolayers were rinsed three times with distilled water for 10 min at room temperature before being stained in 1 mL of filipin for 60 min [14]. The filipin staining solution was prepared by dissolving 2.5 mg of filipin in 1 mL of dimethylformamide. This was then added to 50 μL of PBS. The stained monolayers were rinsed with isopropanol for 1 min and a glass coverslip was mounted over the microscope slide culture with 10% glycerol in PBS. Slides were examined and photographed by fluorescence (ultraviolet light excitation from a 100-watt mercury arc lamp passed through a UG1 filter; fluorescence emission was viewed through a 510-nm barrier filter) and phase microscopy. All fluorescence micrographs were taken using 60-sec exposures.

Protein Determination

Protein concentration was determined using a BCA protein assay reagent from Pierce.

RESULTS

Effect of Azole Antimycotics on Cholesteryl Ester Formation in Macrophages

We previously demonstrated that phosphatidylserine-containing liposomes are effectively endocytosed by cultured macrophages and that the liposomal components, such as phospholipids and cholesterol, are metabolized to form

cholesteryl ester and triacylglycerol in the cells [12, 15]. Using this system, we examined the effects of some azole antimycotics on the formation of cholesteryl ester by macrophages. When the macrophages were incubated with liposomes containing [^{3}H]cholesterol, [^{3}H]cholesteryl ester accumulated progressively in the cells, whereas incubation with 10 μM azole antimycotics such as ketoconazole, econazole and miconazole suppressed cholesteryl ester formation (Fig. 1). As a positive control, melinamide, a known inhibitor of ACAT, completely blocked the cholesteryl ester formation at 3 μM. When macrophages were incubated with liposomes containing a trace amount of 1, 2-di[1-^{14}C]palmitoyl-glycerophosphocholine, the radiolabeled acyl chains were incorporated into triacylglycerol in the cells. All the antimycotics tested had essentially no effect on triacylglycerol formation (Fig. 1B), indicating that these agents do not inhibit either lysosomal hydrolysis of liposomal phospholipids or re-esterification of the acyl chains into triacylglycerol. Ketoconazole exhibited dose-dependent inhibition of cholesteryl ester formation (Fig. 2A); the concentration for half-maximum inhibition was 1–2 μM, and complete inhibition was achieved at 10 μM. Other azole antimycotics, including miconazole and econazole, also suppressed the formation of cholesteryl ester in a dose-dependent manner, but to a lesser extent (Fig. 2, B and C). At the concentrations used, these antimycotics did not affect the uptake of liposomes, indicating that their inhibitory effect is not due to general cytotoxicity. These data indicate that these azole antimycotics specifically inhibit cholesteryl ester formation from endocytosed cholesterol in macrophages. Since the uptake of the liposomes was normal in the presence of azole antimycotics, cholesterol taken up with liposomes should have been present in an unesterified form in the macrophages.

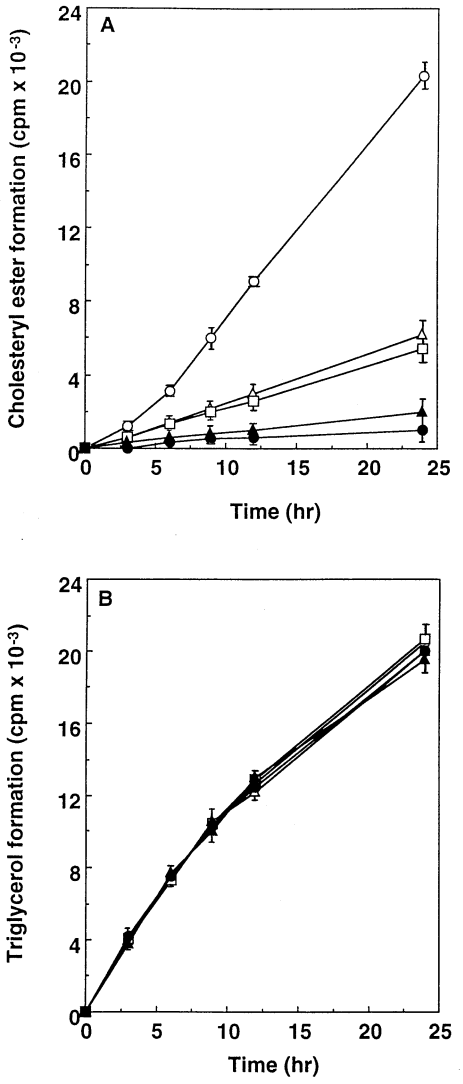

FIG. 1. Effects of azole antimycotics on cholesteryl ester and triacylglycerol formation in macrophages. (A) Macrophage monolayers obtained from 2 × 10^6 peritoneal cells were incubated in 1 mL of medium A with 50 μL of liposomes containing 1 μCi of [^{14}C]cholesterol in the absence (open circles) or presence of ketoconazole (closed triangles), econazole (open triangle), miconazole (open squares) each at 10 μM, or melinamide at 3 μM (closed circles). (B) Macrophage monolayers were incubated in 1 mL of medium A with 50 μL of liposomes containing 1 μCi of 1,2-di[1-^{14}C]palmitoyl-glycerophosphocholine in the absence (open circles) or presence of ketoconazole (closed triangles), econazole (open triangles), and miconazole (open squares) each at 10 μM. After incubation for the indicated time, the macrophages were processed for determination of cellular protein and the radioactivity in the [^{14}C]cholesteryl ester (A) and [^{14}C]triacylglycerol fractions, as described in Materials and Methods. Each value represents the means ± SD of three experiments.

Cytochemical Localization of Endocytosed Cholesterol in the Presence of Ketoconazole

In order to examine the localization of endocytosed cholesterol, light microscopic observation was performed during the metabolism of liposomes by macrophages in the presence of azole antimycotics. Macrophages were first incubated with liposomes for 24 hr and then chased in fresh medium without liposomes, in the presence or absence of 10 μM ketoconazole. As shown in Fig. 3, macrophages that had endocytosed exogenous liposomes in the presence of ketoconazole showed vacuole formation in their cytoplasm. These vacuoles were endocytotic (or phagocytotic), and resulted from uptake of exogenous liposomes, since FITC-derived fluorescence was observed exclusively in these vacuoles upon incubation with liposomes containing entrapped FITC-dextran (data not shown). During chase incubations, the vacuoles disappeared rapidly in the cells incubated in the absence of ketoconazole (Fig. 3A), whereas an appreciable number of vacuoles remained in the cells incubated in the presence of ketoconazole (Fig. 3B). Interestingly, vacuoles were not observed after 12 hr of chase incubation, even in the presence of ketoconazole, when cholesterol-depleted liposomes were used (Fig. 3C). These results suggested that the delay in the disappearance of phagocytotic vacuoles induced by ketoconazole occurred only when the cells endocytosed exogenous cholesterol, and that cholesterol taken up by the cells accumulated in the phagocytotic vacuoles (or phagolysosomes) in ketocon-

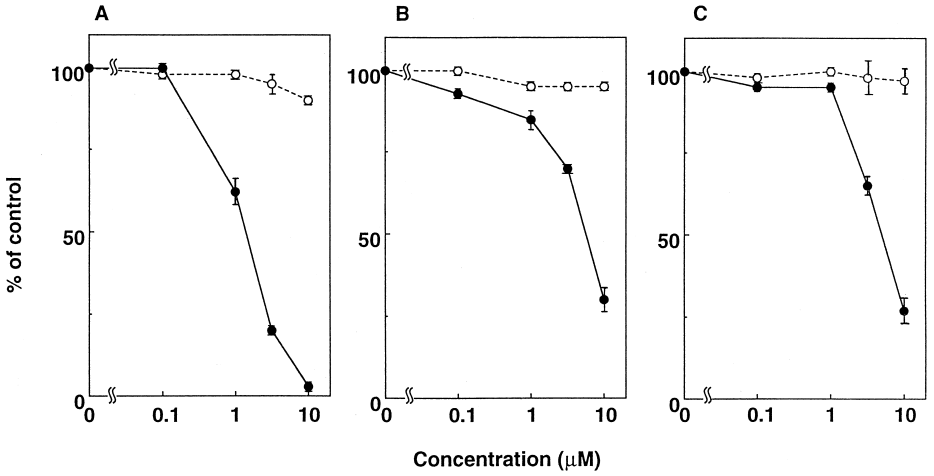

FIG. 2. Dose dependence of azole antimycotic inhibition of cholesteryl ester formation. Macrophage monolayers were incubated in 1 mL of medium A with 50 μL of liposomes containing 1 μCi of [^{14}C]cholesterol in the presence of the indicated concentrations of ketoconazole (A), miconazole (B), or econazole (C). After incubation for 16 hr, each macrophage culture was processed for determination of cellular protein, and the radioactivity associated with the cells (open circles) and that in the cholesteryl ester fraction (closed circles). Each value represents the means ± SD of three experiments.

azole-treated cells. Next, the subcellular localization of endocytosed cholesterol in macrophages was examined using filipin, a fluorescent dye which binds specifically to unesterified cholesterol. After the cells were incubated with liposomes in the absence or presence of ketoconazole, they were stained with filipin and examined by fluorescence microscopy. Cells cultured with liposomes alone were stained uniformly by the dye (Fig. 4A). On the other hand, cells incubated with liposomes and 10 μM ketoconazole exhibited intensive fluorescence in the phagocytotic vacuoles (Fig. 4B), indicating intensive accumulation of endocytosed cholesterol in the phagolysosomes of the macrophages.

Effect of Ketoconazole on 25-Hydroxycholesterol-Stimulated Esterification of Endogenous Cellular Cholesterol

ACAT, an intracellular enzyme responsible for esterifying cholesterol, is known to utilize both exogenously and endogenously derived cholesterol as a substrate [16]. It has been established that the esterification of endogenous cholesterol is stimulated upon incubation of cells with oxygenated sterols such as 25-hydroxycholesterol [17]. To investigate the effect of ketoconazole on the esterification of endogenous cholesterol, we examined 25-hydroxycholesterol-stimulated cholesterol esterification in the presence of ketoconazole. As shown in Fig. 5, 10 μM ketoconazole suppressed 25-hydroxycholesterol-stimulated cholesterol esterification by approximately 35%. On the other hand, 3 μM melinamide inhibited the endogenous cholesterol esterification almost completely. Thus, it was clarified that 10 μM ketoconazole inhibited the esterification of endogenous cholesterol only partially, although under these conditions the esterification of exogenously derived cholesterol was suppressed completely (Figs. 1 and 2).

DISCUSSION

In the present study, we demonstrated that ketoconazole, an azole antimycotic, effectively blocked cholesteryl ester formation by cultured macrophages. Ketoconazole did not impair processes such as the uptake of liposomes or triacylglycerol formation, and instead inhibited cholesteryl ester formation in macrophages by blocking the transport of endocytosed cholesterol from lysosomes to the endoplasmic

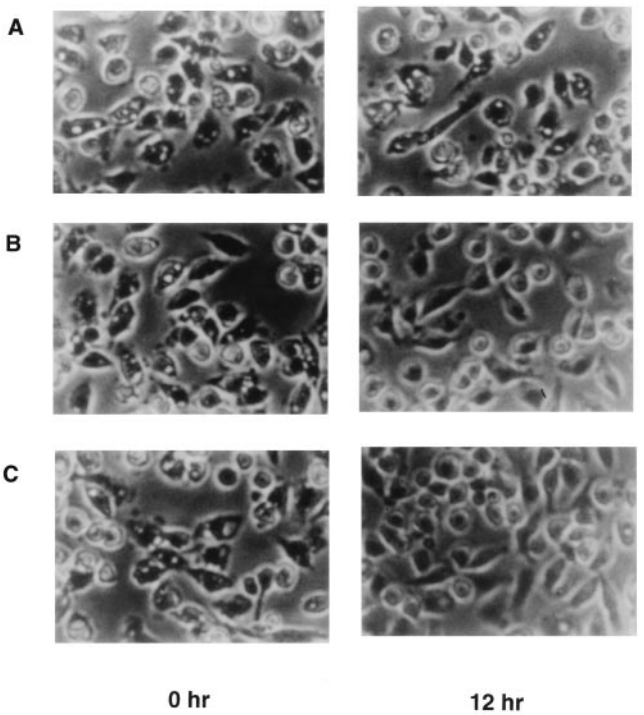

FIG. 3. Morphological examination of macrophages taking up liposomes in the presence of ketoconazole. Macrophage monolayers were incubated for 18 hr in the presence of 10 μM ketoconazole in 1 mL of medium A with 50 μL of liposomes composed of phosphatidylcholine (50 nmol), phosphatidylserine (50 nmol), cholesterol (75 nmol), and dicetylphosphate (10 nmol) (A, B), or with 50 μL of liposomes composed of phosphatidylcholine (50 nmol), phosphatidylserine (50 nmol), and dicetylphosphate (10 nmol) (C). Then the cells were washed three times with medium A, re-fed 1 mL of medium A containing (A, C) or not containing (B) 10 μM ketoconazole, and incubated for 12 hr. Macrophages were examined by light microscopy at × 200 magnification after chase incubation for 0 and 12 hr.

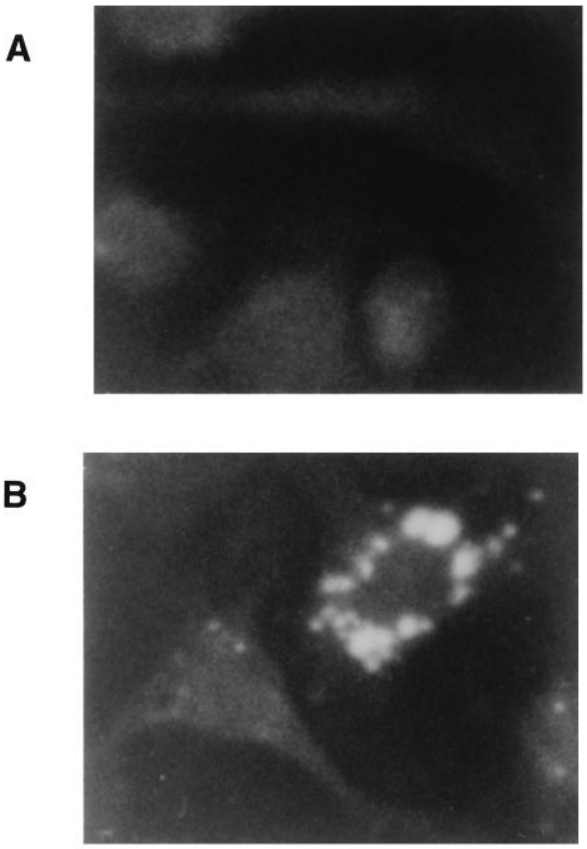

FIG. 4. Cytochemical localization of unesterified cholesterol in ketoconazole-treated macrophages by filipin staining. Macrophage monolayers grown in microscope slide chambers (0.5 mL) were incubated with 25 μL of liposomes for 24 hr in the absence (A) and presence (B) of 10 μM ketoconazole. The monolayers were then washed with HBSS. Fixation and staining with filipin were performed as described in Materials and Methods (magnification, ×400).

reticulum. This conclusion was based on the observations that: 1) ketoconazole completely inhibited the esterification of endocytosed cholesterol at concentrations where the ACAT reaction (as revealed by endogenous cholesterol esterification) was only partially impaired; and 2) ketoconazole restricted endocytosed cholesterol to within the lysosomal compartment.

Recent evidence indicates that the movement of cholesterol from lysosomes to other cellular sites is neither random nor diffuse, but is precisely regulated [18]. The existence of naturally occurring [19] and drug-induced cell lines [20, 21] with defective lysosomal cholesterol transport suggests that a cellular protein(s) is/are involved in lysosomal cholesterol transport. Moreover, agents which inhibit cholesterol efflux from lysosomes have been reported by several groups. We have demonstrated that a series of structurally related steroids characterized by the presence of an oxo group at the C-17 or C-20 position, such as progesterone, pregnenolone, androstenedione and dehydroisoandrosterone, specifically inhibit the transport of lysosomal cholesterol in cultured macrophages [15]. Butler *et al.* have also reported that progesterone and pregnenolone induce extensive accumulation of cholesterol in lysosomes with cultured fibroblasts [22]. Indeed, similar morphological changes, such as accumulation of phagolysosomes and restriction of unesterified cholesterol in them, which we observed with ketoconazole, were also obtained with macrophages upon treatment with these inhibitors [15]. It is also noted that macrophages treated with a specific ACAT inhibitor do not exhibit these morphologies upon incubation with cholesterol-containing liposomes [15]. Hydrophobic amines, such as U18666A [23], imipramine [24], stearylamine and sphinganine [25], are also reported to suppress lysosomal cholesterol transport. The findings of the present study indicate that ketoconazole is another in this series of inhibitors of lysosomal cholesterol transport in mammalian cells.

It is well known that ketoconazole suppresses cholesterol biosynthesis by inhibiting 14α-demethylation of lanosterol in mammalian cells. Gupta *et al.* [26] have studied in detail the effect of ketoconazole on cholesterol metabolism using rat intestinal epithelial cell cultures. They observed that at lower concentrations (0.15–2 μM) ketoconazole caused concentration-dependent inhibition of HMG-CoA reductase activity with concomitant accumulation of polar sterols such as 24, 25-epoxylanosterol and their metabolites. Favata *et al.* [27] reported that formation of these oxysterol intermediates is necessary and sufficient for modulating

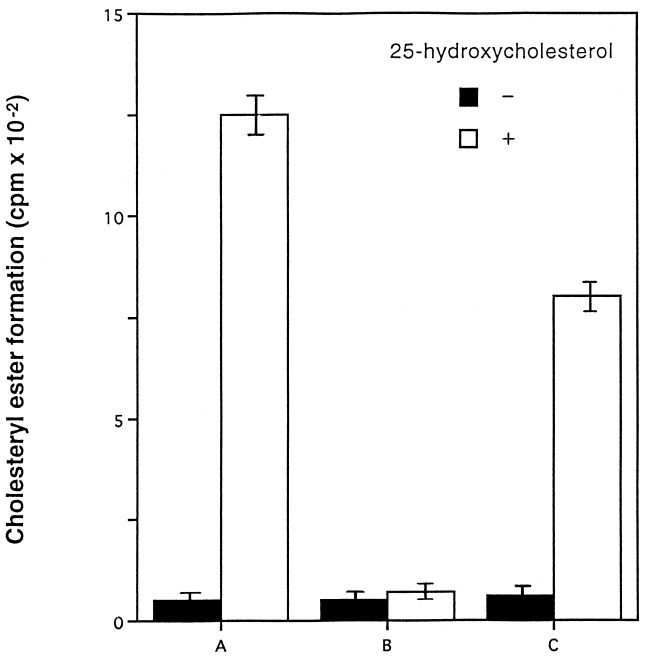

FIG. 5. Effect of ketoconazole on 25-hydroxycholesterol-stimulated esterification of endogenous cholesterol. Macrophage monolayers were incubated in 1 mL of medium A containing 50 μL of BSA–[^{3}H]oleate complex (see Materials and Methods) and 6 μM 25-hydroxycholesterol in the absence (A) and presence of 3 μM melinamide (B) or 10 μM ketoconazole (C). After incubation for 12 hr, each macrophage culture was processed for determination of cellular protein and the radioactivity in the cholesteryl ester fraction. Each value represents the means ± SD of three experiments.

HMG-CoA reductase when azole antimycotic drugs are used. Thus, ketoconazole suppresses cholesterol biosynthesis by two different mechanisms in mammalian cells: direct inhibition of the cholesterol-biosynthetic enzyme, and production of secondary bioactive sterol analogs such as oxygenated sterols from cholesterol precursors, by which HMG-CoA reductase is down-regulated. As mentioned above, lysosomal cholesterol transport is inhibited by some oxysterols such as progesterone and pregnenolone. It is still unknown whether ketoconazole directly inhibits lysosomal cholesterol transport, or produces secondary mediators such as oxgenated sterols capable of inhibiting this process in cultured macrophages. However, our preliminary observations suggest that ketoconazole exerts its inhibitory effect on cholesteryl ester formation even in the presence of compactin, an inhibitor of HMG-CoA reductase, indicating that mevalonate-derived, endogenously generated oxysterols are not required for the inhibition.

Ketoconazole is reported to prevent LDL-mediated suppression of both HMG-CoA reductase and LDL receptor expression [28]. It has also been demonstrated previously [29] that suppression of HMG-CoA reductase by LDL does not require mevalonate synthesis and hence mevalonate-derived oxysterol, indicating that ketoconazole does not work through an endogenously generated oxysterol pathway. Cells treated with drugs that inhibit lysosomal cholesterol transport, or mutant cells with defective lysosomal cholesterol transport, have a defect in LDL-mediated suppression of cholesterol synthesis and LDL receptor down-regulation [30, 31]. Taken together with the present data, it is reasonable to assume that ketoconazole prevents the LDL-dependent suppression of these events by inhibiting cholesterol transport from lysosomes.

In conclusion, ketoconazole has multiple effects on cholesterol metabolism in mammalian cells, including inhibition of cholesterol biosynthesis and the ACAT reaction, and stimulation of polar and possibly oxygenated sterols that may regulate cholesterol metabolism. We have also clarified that ketoconazole has an additional effect of inhibiting lysosomal cholesterol transport at a concentration of 10 μM. Care must be exercised in concluding which effect is the dominant one in experiments employing ketoconazole.

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