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Regulation of hepatic cholesterol biosynthesis

Effects of a cytochrome *P*-450 inhibitor on the formation and metabolism of oxygenated sterol products of lanosterol

José IGLESIAS* and Geoffrey F. GIBBONS†
Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

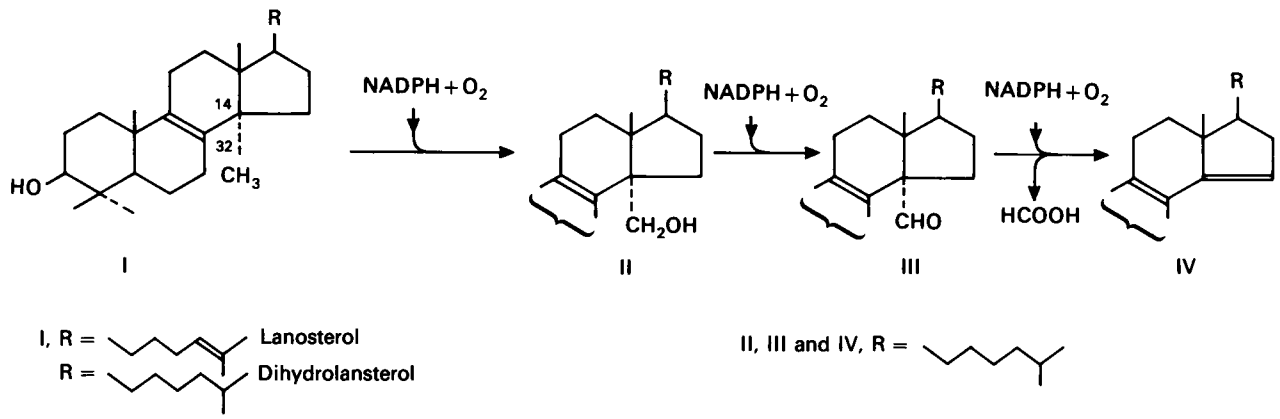
The involvement of oxygenated cholesterol precursors in the regulation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity was studied by examining the effect of ketoconazole on the metabolism of mevalonic acid, lanosterol and the lanosterol metabolites, lanost-8-ene-3β,32-diol, 3β-hydroxylanost-8-en-32-al and 4,4-dimethylcholesta-8,14-dien-3β-ol, in liver subcellular fractions and hepatocyte cultures. Inhibition of cholesterol synthesis from mevalonate by ketoconazole at concentrations up to 30 μM was due exclusively to a suppression of cytochrome $P\text{-}450_{LDM}$ (LDM = lanosterol demethylase) activity, resulting in a decreased rate of lanosterol 14α-demethylation. No enzyme after the 14α-demethylase step was affected. When [^{14}C]mevalonate was the cholesterol precursor, inhibition of cytochrome $P450_{LDM}$ was accompanied by the accumulation of several labelled oxygenated sterols, quantitatively the most important of which was the C-32 aldehyde derivative of lanosterol. There was no accumulation of the 24,25-oxide derivative of lanosterol, nor of the C-32 alcohol. Under these conditions the activity of HMG-CoA reductase declined. The C-32 aldehyde accumulated to a far greater extent when lanost-8-ene-3β,32-diol rather than mevalonate was used as the cholesterol precursor in the presence of ketoconazole. With both precursors, this accumulation was reversed at higher concentrations of ketoconazole in liver subcellular fractions. A similar reversal was not observed in hepatocyte cultures.

INTRODUCTION

One of the means by which cells compensate for changes in cholesterol balance involves modification of the rate of endogenous cholesterol synthesis. This is brought about mainly by changes in the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34), which regulates carbon flux into the cholesterol pathway [1]. However, the exact sequence of events by which cellular cholesterol status is transmitted to HMG-CoA reductase is poorly understood. Early studies by Kandutsch and his colleagues provided strong evidence that oxygenated cholesterol derivatives may act as second messengers in this process [2–4]. Sterols of this type occur naturally during cholesterol biosynthesis [1,5,6], but it is only quite recently that changes in the amounts of these substances have been correlated with changes in the activity of HMG-CoA reductase. These studies involved, mainly, the use of inhibitors of the triterpenoid part of the cholesterol pathway. For instance, inhibition of squalene-2,3-oxide cyclase (EC 5.4.99.7) by the drug U-18666A in intestinal epithelial cells resulted in the formation of squalene-2,3:22,23-dioxide, which was then cyclized by residual enzyme activity to 24,25-oxidolanosterol, which accumulated [7,8]. Similarly, inhibition of lanosterol 14α-demethylation by ketoconazole in liver resulted in increased amounts of the C-32 oxygenated intermediates of the demethylation sequence lanost-8-ene-3β,32-diol and 3β-hydroxylanost-8-en-32-al (structures II and III, Scheme 1) [9,10]. In both cases, there was a decline in the activity of HMG-CoA reductase. The experiments using ketoconazole were of particular interest, since this drug is an inhibitor of cytochrome *P*-450 [11], an isoenzyme of which is essential for the oxidative 14α-demethylation of lanosterol during cholesterol biosynthesis [12–15]. The specificity of enzyme inhibition by ketoconazole, however, has not been clearly defined, and it has been reported that in non-hepatic cells the drug also causes the appearance of other oxygenated sterols, notably the 24,25-oxido derivative of lanosterol [16,17]. This is convertible into 24,25-oxidocholesterol, a known suppressor of HMG-CoA reductase activity [8]. Similarly, the work of Miettinen has suggested that, with high concentrations in human subjects, the drug interferes with post-lanosterol enzymes of the cholesterol pathway [18]. An objective of the present work was to clarify the effects of ketoconazole on enzymic processes producing oxysterols in the liver and to correlate these effects with HMG-CoA reductase activity. Also, in view of increasing awareness of the possible role of 3β-hydroxylanost-8-en-32-al as a natural regulator of HMG-CoA reductase activity [10,17,19,20], another aim of this work was to examine the kinetics of formation and further metabolism of this obligatory cholesterol precursor under conditions known to produce changes in reductase activity. These studies

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

* Present address: Department of Biochemistry and Molecular Biology, University of Granada, Granada, Spain.
† To whom reprint requests should be addressed.



Scheme 1. Intermediates in the removal of the 14α-methyl group (C-32) during cholesterol biosynthesis

were facilitated by the availability of radiochemically labelled lanost-8-ene-3β,32-diol and 3β-hydroxylanost-8-en-32-al prepared from lanosterol by chemical synthesis [19,21].

MATERIALS AND METHODS

Maintenance of animals

Male Wistar rats were kept in a windowless room lit artificially with tungsten bulbs between 16:00 h and 04:00 h for at least 2 weeks before use. During this period the rats were fed on a commercially available pelleted diet (Diet PRM; E. Dixon and Sons, Ware, Herts., U.K.), which contained (w/w) 47.5% carbohydrate (mainly starch), 2.7% fat and 18.7% protein. Food and water were available *ad libitum*. Animals weighing between 200 and 270 g were used for experiments.

Preparation of hepatocyte cultures and assay of HMG-CoA reductase

Hepatocytes were isolated under sterile conditions and suspended in Waymouth's medium MB 752/1 containing fetal-calf serum (10%, v/v) and other supplements as described previously [22]. At 4 h after the suspension was plated out into dishes, the medium was changed and the cell monolayer was washed three times with the above medium lacking serum. Serum-free medium (3.0 ml) was then added to each dish. [2-^{14}C]Mevalonate was added as a solution in Waymouth's medium. Labelled sterol substrates were emulsified as described below (except that Waymouth's medium was used instead of potassium phosphate buffer) and added as a solution in Waymouth's medium (50 μl) containing Tween 80 (0.5%). The dishes were then incubated in a humidified atmosphere of air/CO_2 (19:1) at 37 °C.

For measurement of HMG-CoA reductase activity the medium was removed and the cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (pH 7.4). The cells were scraped from the dish into 2.0 ml of the same buffer and sedimented by centrifugation (1.5 min at 80 *g*). The buffer was removed and the cells were resuspended in 1.0 ml of 50 mM-potassium phosphate buffer (pH 7.4) containing dithiothreitol (5 mM) and EDTA (1 mM). The suspension was sonicated at 6 μm amplitude for 10 s, and enzyme activity was determined as described by Cavenee *et al.* [23].

Incubation of cholesterol precursors with a subcellular fraction of rat liver

Livers were removed from sodium pentobarbital-anaesthetized animals and immediately chilled on ice. Each liver was minced through an ice-cold stainless-steel tissue mincer, and a post-mitochondrial supernatant (16000 *g* supernatant, designated S_{16}) was obtained by the method of Bucher *et al.* [24]. Portions of this supernatant (2.0 ml) were used as the source of enzymes for investigating the metabolism of [2-^{14}C]mevalonate, [^{14}C]lanosterol, 5α-[2-^{3}H]lanost-8-ene-3β,32-diol, 3β-hydroxy-5α-[2-^{3}H]lanost-8-en-32-al and 4,4-dimethyl-5α-[2-^{3}H]cholesta-8,14-dien-3β-ol. The steroidal substrates were emulsified by dissolving in 0.25% Tween 80 solution in acetone, evaporating the solvent and resuspending the residue in 0.25% Tween 80 solution in 0.1 M-potassium phosphate buffer (pH 7.4). The final Tween/sterol ratio was 33:1 (w/w). Cofactors were added as stated in the legends to the Figures.

Isolation of labelled sterols

After incubation, known amounts of [^{3}H]cholesterol and [^{14}C]cholesterol were added as internal standards to incubations containing the ^{14}C-labelled and ^{3}H-labelled substrates respectively. In addition, non-labelled squalene, dihydrolanosterol, lanosterol, cholesterol, 25-hydroxycholesterol (500 μg of each) and, in some cases, 24,25-oxidolanosterol (100 μg) were added as carriers, and the non-saponifiable-lipid fraction was obtained as described previously [25]. Diethyl ether was used for the lipid extraction. The individual components of the non-saponifiable fraction were isolated by t.l.c. on silica-gel G with chloroform (System 1). This separated cholesterol and companion C_{27} sterols (R_F 0.26) from lanosterol and companion C_{30} and C_{29} sterols (R_F 0.38). Squalene had an R_F of 0.84. Fractions containing very polar sterols (R_F 0.00–0.07) and polar sterols (R_F 0.09, containing 25-hydroxycholesterol and lanost-8-ene-3β,32-diol) were also scraped from the plate. All fractions except those containing the polar sterols (R_F 0.09) and cholesterol (R_F 0.26) were scraped directly into scintillation vials and assayed for ^{3}H or ^{14}C.

Previous experiments had shown that t.l.c. System 1 was incapable of separating cholesterol efficiently from the C-32 aldehyde. 24,25-Oxidolanosterol also has a mobility identical with that of the C-32 aldehyde in this system. These three sterols were separated after elution

of the silica-gel obtained in t.l.c. System 1 with diethyl ether. After evaporation of the ether, the sterol fraction was acetylated, followed by t.l.c. on silica-gel G with hexane/diethyl ether (4:1, v/v) as the solvent (System 2). The R_F values of the 3β-acetates of cholesterol, the C-32 aldehyde and 24,25-oxidolanosterol were 0.47, 0.33 and 0.28 respectively. To test the efficiency of this system in separating the last two sterols, a mixture of the acetates of the 2-^{3}H-labelled aldehyde and the unlabelled 24,25-oxide were chromatographed. On average, only $11.1 \pm 2.2\%$ (S.E.M., $n = 6$) of the recovered radioactive aldehyde occurred in the fraction containing the 24,25-oxide.

The labelled components of the polar sterol fraction in System 1 (R_F 0.09) were also examined after their elution with diethyl ether. In this case, the labelled material was further chromatographed on silica-gel G with ether/hexane (7:3, v/v) as the solvent (System 3). 25-Hydroxycholesterol, lanost-8-ene-3β,32-diol and cholesterol had R_F values of 0.20, 0.26 and 0.40. At every stage of the extraction and purification procedure, and during storage of samples, air-oxidation of sterols was minimized by addition of butylated hydroxytoluene (2 μg/ml of solvent).

Materials

[^{14}C]Lanosterol was prepared biosynthetically from [2-^{14}C]mevalonate as described previously [12], except that ketoconazole (140 μM) was used instead of CO to prevent further metabolism into cholesterol. It was extensively purified in several t.l.c. systems before use [12]. Its specific radioactivity was 7310 d.p.m./nmol. [2-^{3}H]Lanost-8-ene-3β,32-diol and 3β-hydroxy[2-^{3}H]lanost-8-en-32-al were prepared by chemical synthesis as described previously [19,21]. They were purified immediately before use by t.l.c. (System 1). Their specific radioactivities were 7600d.p.m./nmol. 4,4-Dimethyl[2-^{3}H]cholesta-8,14-dien-3β-ol was synthesized from cholesta-5,7-dien-3β-ol as described previously [14,26]. It was purified immediately before use by t.l.c. in System 1. Its specific radioactivity was 5799 d.p.m./nmol. 24,25-Oxidolanosterol, a mixture of 24(R)- and 24(S)-epimers, with the former predominating, was given by Dr. Thomas Spencer, Dartmouth College, Hanover, NH, U.S.A. Ketoconazole was given by Mr. N. Blatchford, Janssen Pharmaceuticals, Wantage, Berks., U.K. All organic solvents were distilled before use and, in addition, diethyl ether was passed through a column of alumina (Grade I; Woelm, Eschwege, Germany) to remove any peroxides. Radiochemicals other than those described above were obtained from Amersham International, Little Chalfont, Bucks., U.K. Tissue-culture media were obtained from Gibco (Paisley, Scotland, U.K.). Enzymes and cofactors were obtained from Sigma (U.K.). Thin-layer plates of silica-gel G and Alumina G were obtained from Anachem (Luton, Beds., U.K.).

RESULTS

Effects of ketoconazole on sterol formation from [2-^{14}C]mevalonate

In S_{16} fractions derived from liver, increasing concentrations of ketoconazole gave rise to an increase in the accumulation of labelled lanosterol, which was accompanied by the decline in the rate of labelled-cholesterol synthesis (Fig. 1). However, the drug had opposite

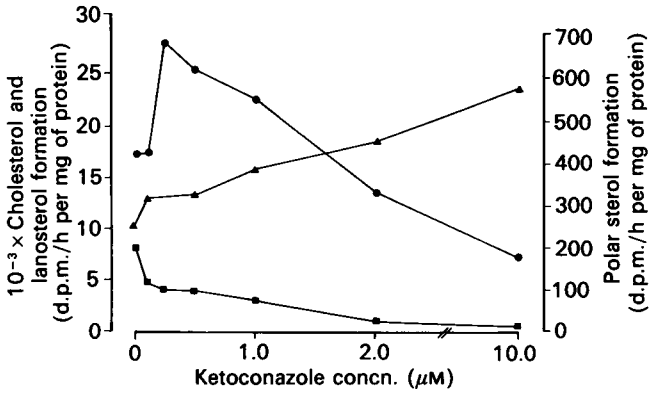


Fig. 1. Effect of ketoconazole on sterol synthesis from [2-^{14}C]mevalonate in a cell-free preparation of liver

A 16000 g supernatant fraction (S_{16}) of rat liver (2.0 ml) was incubated with different concentrations of ketoconazole in 0.1 M-HCl (50 μl) for 45 min at 37 °C. Control incubations contained 0.1 M-HCl (50 μl) only. Each incubation contained sucrose (0.125 M), GSH (10 mM), nicotinamide (30 mM), EDTA (1 mM), $MgCl_2$ (4 mM), fructose 1,6-bisphosphate (16 mM), NAD^+ (1.6 mM) and 0.1 M-potassium phosphate buffer (pH 7.4) in a total volume of 2.5 ml. Incubations were begun by the addition of (R)-[2-^{14}C]mevalonate (1 μCi) and were stopped by the addition of 10.0 ml of ethanolic 7.5% (v/v) KOH. The various labelled sterol fractions were isolated as described in the Materials and methods section: ▲, labelled lanosterol; ■, labelled cholesterol; ●, labelled polar sterols.

effects on the accumulation of labelled polar sterols (i.e. total sterols of polarity greater than cholesterol), depending on its concentration. At low concentrations (0.1–1.0 μM) the amounts of these labelled substances increased, whereas at higher drug concentrations (> 1.0 μM) there was a decline. Subsequent experiments showed that these sterols could be sub-divided by t.l.c. into two classes, the most abundant of which had the greater polarity (R_F 0.00–0.07). The other fraction (R_F 0.07–0.20) had a mobility similar to 25-hydroxycholesterol and lanost-8-ene-3β,32-diol. These fractions were not investigated further in these experiments. In a similar experiment, the amounts of the labelled 32-aldehyde and 24,25-oxido derivatives of lanosterol formed from [2-^{14}C]mevalonate in the presence of ketoconazole were determined. In this case, manipulative losses were accounted for by inclusion of a known amount of the 2-^{3}H-labelled C-32 aldehyde as internal standard at the end of each incubation. The patterns of incorporation of labelled mevalonate are shown in Fig. 2. Low concentrations of ketoconazole resulted in an increase in the amounts of the ^{14}C-labelled C-32 aldehyde, an effect which was reversed at higher concentrations of the drug. Subsequent experiments showed that there was a considerable variation in the concentration of ketoconazole required for maximum accumulation of the C-32 aldehyde and, in some cases, increases occurred up to a concentration of 10 μM. Ketoconazole had no effect on the amounts of label associated with 24,25-oxidolanosterol.

The effects of ketoconazole on the metabolism of [2-^{14}C]-mevalonate in hepatocyte cultures are shown in Fig. 3. In general, higher concentrations of the drug were required to obtain similar effects to those observed in the

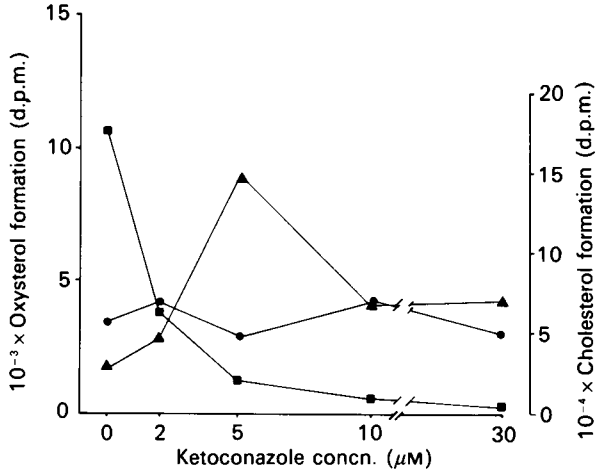


Fig. 2. Effect of ketoconazole on the formation of 3β-hydroxylanost-8-en-32-al and 24,25-oxidolanosterol

S_{16} fractions of rat liver were incubated with ketoconazole and *R*-[2-^{14}C]mevalonate (1.5 μCi) as described above. The sterol fractions were isolated as described in the Materials and methods section: ▲, labelled 3β-hydroxylanost-8-en-32-al; ●, labelled 24,25-oxidolanosterol; ■, labelled cholesterol.

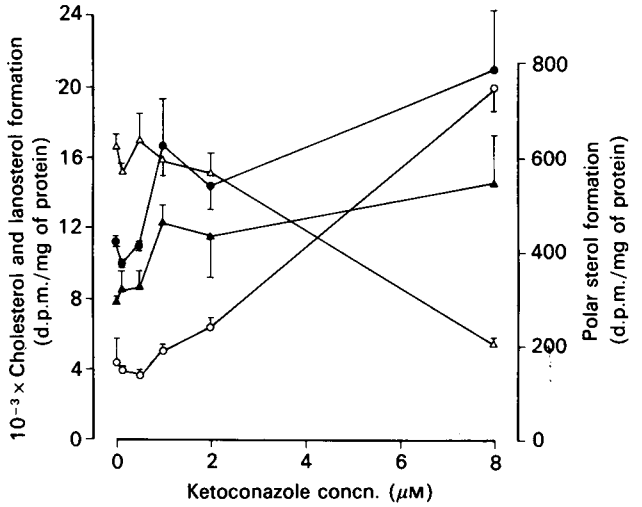


Fig. 3. Effect of ketoconazole on the conversion of [2-^{14}C]-mevalonate into cholesterol, lanosterol and polar sterols by cultured hepatocytes

Hepatocytes were plated out in serum-containing Waymouth's medium. After 4 h, the medium was removed, washed three times with serum-free Waymouth's medium and 3.0 ml of the serum-free medium was added to each dish. *R*-[2-^{14}C]Mevalonate (1 μCi) was added to dishes containing different concentrations of ketoconazole added as a solution in 0.1 M-HCl (50 μl). All incubations were carried out for 18 h at 37 °C. Cells were washed and the sterol fractions were isolated as described in the Materials and methods section: ○, lanosterol-containing fraction (R_F 0.38); △, cholesterol-containing fraction (R_F 0.26); ▲, polar sterols (R_F 0.07–0.20); ●, very polar sterols (R_F 0.00–0.07). In this system 25-hydroxycholesterol had an R_F of 0.09. Each incubation was carried out in triplicate, and the values represent means ± S.E.M.

subcellular fractions. However, ketoconazole again led to an increase in the amounts of label associated with the lanosterol-containing fraction at the expense of labelled cholesterol. Argentation chromatography of the lanosterol-containing fraction [12] showed that 67% of the radioactivity was associated with lanosterol, 28% with 24,25-dihydrolanosterol and less than 5% with their 14α-demethyl derivatives (C_{29} sterols). This confirms that the major site of blockage was at the 14α-demethylation step. These effects were not very pronounced at drug concentrations < 2 μM, in contrast with the pattern observed with the subcellular fraction (Fig. 1). Nevertheless, in the cell cultures over this low concentration range there were increases in the accumulation of label in two fractions more polar than cholesterol ($P < 0.01$ for the least pollar of these fractions).

Because of the decreased effectiveness of ketoconazole in the hepatocyte culture system, other experiments were carried out at higher concentrations of the drug (Fig. 4). In this case the labelled contents of the oxysterol-containing fractions were investigated more thoroughly. First, the labelled C-32 aldehyde was purified from the cholesterol-containing fraction by acetylation, followed by t.l.c. in System 2. Second, the labelled fraction of R_F 0.07–0.20 was further resolved (t.l.c. System 3) into two separate fractions, with the mobilities of 25-hydroxycholesterol (R_F 0.20) and lanost-8-ene-3β,32-diol (R_F 0.26). Of the oxygenated lanosterol metabolites, only the C-32 aldehyde contained appreciable amounts of label, the quantities of which increased in an almost linear fashion up to a drug concentration of 30 μM (Fig. 4*b*). At this concentration, the label in the C-32 aldehyde amounted to 56% of that in cholesterol and 4% of that in the lanosterol-containing fraction. In contrast with the cell-free system, no biphasic effect of ketoconazole on the labelling of the C-32 aldehyde was observed in the hepatocyte cultures. This may reflect the decreased effectiveness of the drug in whole cells compared with the cell-free model. Concentrations of ketoconazole greater than 30 μM were visibly toxic to the cells, so no attempts were made to study sterol accumulation in a higher concentration range. Very little label was associated with the C-32 alcohol, the immediate precursor of the C-32 aldehyde, at any concentration of ketoconazole. Most of the radioactivity of the original fraction from which it was derived (R_F 0.07–0.20, System 1), corresponded, on subsequent t.l.c., to a fraction with the chromatographic mobility of 25-hydroxycholesterol. The amount of this labelled material reached a maximum at a drug concentration of 5 μM (Fig. 4*b*). The small amounts of radioactivity precluded any further investigation into the nature of this substance. Ketoconazole again resulted in an increase in the incorporation of label into a very polar sterol fraction (R_F 0.00–0.07, System 1). This amount reached a peak at 5 μM-ketoconazole. Although relatively low concentrations of the drug always gave rise to an increase in the accumulation of label in this fraction, the exact concentration required for maximum labelling varied. In four independent experiments, two reached a maximum at 5 μM-ketoconazole and two at 10 μM.

Response of HMG-CoA reductase to ketoconazole

Treatment of cells with an increasing concentration of ketoconazole gave rise to a decrease in the activity of HMG-CoA reductase measured 6 h later (Fig. 5). Ketoconazole had no direct effect on HMG-CoA reductase

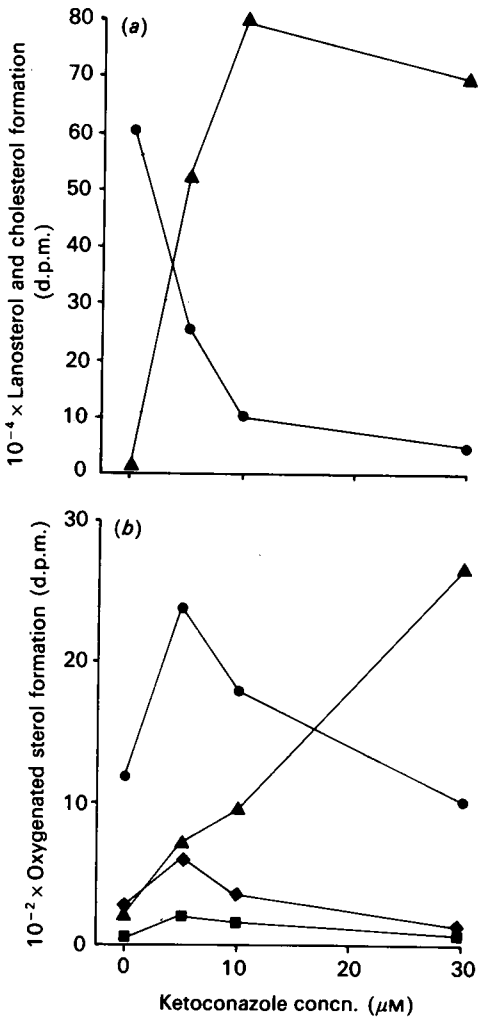


Fig. 4. Characterization of polar sterols formed in the presence of ketoconazole in cultured hepatocytes

Hepatocytes were incubated with *R*-[2-^{14}C]mevalonate (0.75 μCi) and ketoconazole as described in the legend to Fig. 3. The labelled sterol fractions were isolated as described in the text. (*a*): ●, Labelled cholesterol; ▲, labelled lanosterol. (*b*): ●, Very polar sterols (R_F 0.00–0.07; System 1); ▲, 3β-hydroxylanost-8-en-32-al; ■, lanost-8-ene-3β,32-diol; ◆, 25-hydroxycholesterol fraction. Dishes were incubated in duplicate, and each point is an average of the two values.

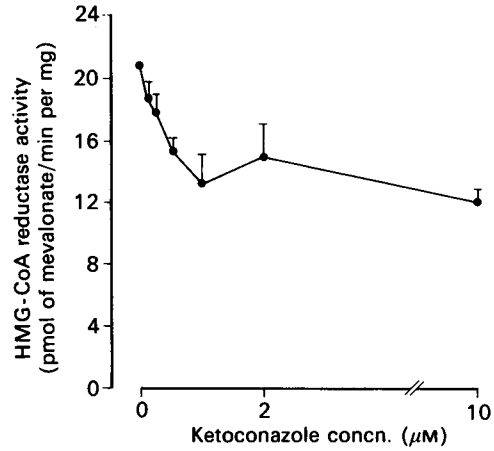


Fig. 5. Effect of ketoconazole on HMG-CoA reductase activity in cultured hepatocytes

Hepatocytes were plated out in serum-containing Waymouth's medium. After 4 h, the medium was removed, the cells were washed three times with serum-free Waymouth's medium and 3.0 ml of the same medium was added to each dish. The cells were incubated overnight at 37 °C, and different concentrations of ketoconazole in 0.1 M-HCl (50 μl) were then added to each dish; 6 h later the cells were harvested and HMG-CoA reductase activity was determined. Each value represents the mean ± S.E.M. of triplicate incubations at each ketoconazole concentration. Enzyme activities are calculated on the basis of total cell protein.

activity when present in the assay mixture at 140 μM (results not shown). The ability of ketoconazole to inhibit HMG-CoA reductase activity in the intact cells appeared to be related to its ability to enhance the accumulation of labelled oxygenated sterols and of labelled lanosterol from [2-^{14}C]mevalonate.

Metabolism of steroidal cholesterol precursors: effects of ketoconazole

Lanosterol 14α-demethylation is a complex process, consisting of several oxidative steps [14,15,27]. To obtain more information about the exact sites inhibited by ketoconazole, we studied the effects of the drug on the metabolism of the substrate (lanosterol), an intermediate (lanost-8-ene-3β,32-diol) and on the product (4,4-dimethylcholesta-8,14-dien-3β-ol) of the 14α-demethylase enzyme in liver subcellular fractions. These were compared with the effects on the metabolism of mevalonate. Fig. 6(*b*) shows that the pattern of inhibition of cholesterol formation from lanosterol was almost identical with that from mevalonate, as was the extent of accumulation of label in the C-32 aldehyde (Fig. 6*a*) and in the amounts of lanosterol remaining at the end of the incubation (Fig. 6*c*). However, the conversion of lanost-8-ene-3β,32-diol into cholesterol was less sensitive to inhibition by ketoconazole, and this was reflected by the smaller quantities of labelled substrate remaining at the end of the incubation (Fig. 6*c*). Nevertheless, the relative accumulation of label in the C-32 aldehyde from this substance was much enhanced by ketoconazole, and at the highest concentration of the drug this amounted to almost 10% of the total radioactivity recovered. Ketoconazole had no effect on the metabolism of the product of lanosterol 14α-demethylase, 4,4-dimethylcholesta-8,14-dien-3β-ol (structure IV, Scheme 1), thereby confirming that, in liver, of all the steroidal steps of cholesterol biosynthesis, ketoconazole at concentrations up to 10 μM interferes exclusively with that requiring cytochrome *P*-450, i.e. lanosterol 14α-demethylation.

In view of the relative decline in the accumulation of the labelled C-32 aldehyde at high concentrations of ketoconazole in a liver S_{16} fraction (Fig. 2) when mevalonate was the cholesterol precursor, it was of interest to determine whether the oxygenated cholesterol precursor, lanost-8-ene-3β,32-diol, behaved in a similar way. This was tested by incubating the diol at drug concentrations up to 30 μM (Fig. 7). Again, concentrations greater than 10 μM reversed the accumulation of label in the C-32 aldehyde.

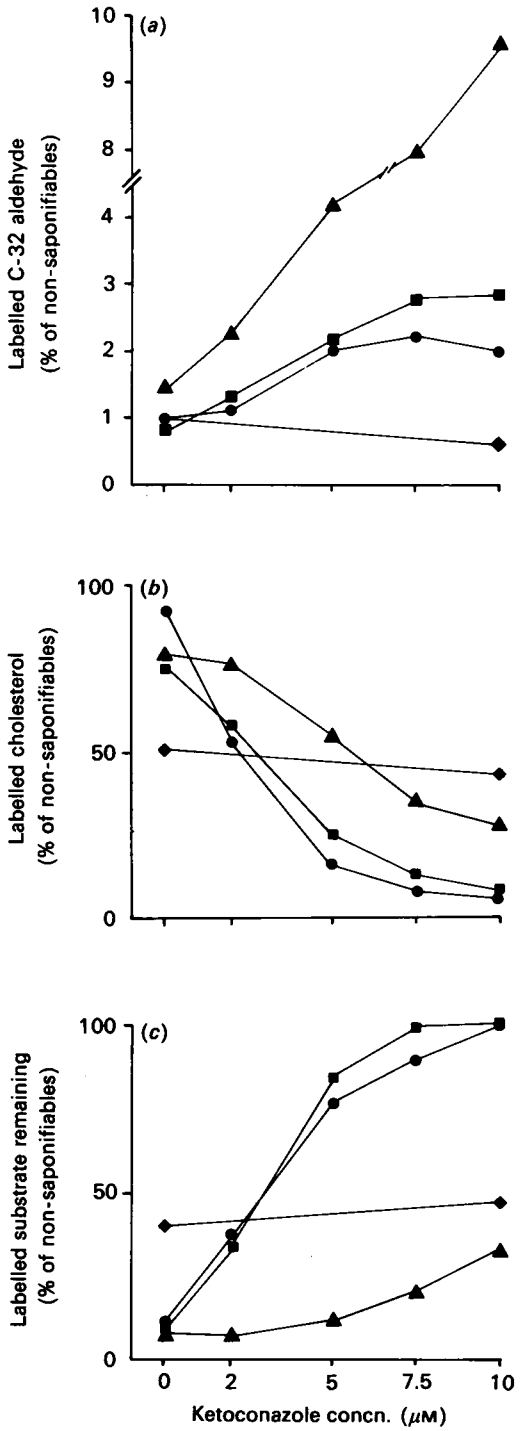


Fig. 6. Metabolism of pre- and post-lanosterol precursors of cholesterol: comparison of the effects of ketoconazole

Post-mitochondrial (16000 *g*) supernatants were prepared from rat liver (see Fig. 1) and incubated with *R*-[2-^{14}C]mevalonate (0.75 μCi), [^{14}C]lanosterol (64530 d.p.m., 9.1 nmol), [2-^{3}H]lanost-8-ene-3β,32-diol (84000 d.p.m., 10.9 nmol) or 4,4-dimethyl[2-^{3}H]cholesta-8,14-dienol (26000 d.p.m., 4.5 nmol) for 45 min. Sterol fractions were isolated as described in the text. Figs. 6(*a*) and 6(*b*) show the amounts of the C-32 aldehyde and cholesterol respectively formed from mevalonate (■), lanosterol (●) lanost-8-ene-3β,32-diol (▲) and 4,4-dimethylcholesta-8,14-dienol (◆). Fig. 6(*c*) shows the amounts of substrates remaining. Symbols are as above, except that ■ shows the amount of lanosterol formed from mevalonate.

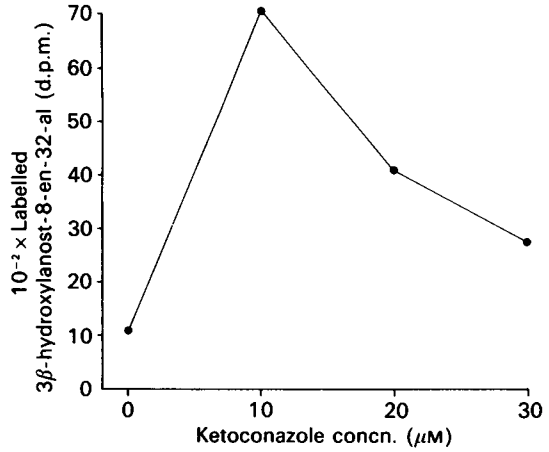


Fig. 7. Biphasic effect of ketoconazole on the formation of 3β-hydroxylanost-8-en-32-al from lanost-8-ene-3β,32-diol

Post-mitochondrial (16000 *g*) supernatants (S_{16}) were prepared as described in Fig. 1 and incubated with [2-^{3}H]lanost-8-ene-3β,32-diol (79500 d.p.m., 10.3 nmol) for 45 min. Ketoconazole was added as a solution in 0.1 M-HCl. Labelled 3β-hydroxylanost-8-en-32-al was isolated as described in the text.

DISCUSSION

Of all the known enzymes of the triterpenoid part of the cholesterol pathway in liver, ketoconazole inhibited only lanosterol 14α-demethylase. Previous work using non-hepatic cells [16,17] showed that the drug also inhibited squalene-2,3-oxide cyclase (lanosterol synthase; EC 5.4.99.7). This gave rise, at low concentrations of the drug, to an increase in the accumulation of 24,25-oxidolanosterol. Ketoconazole, at least up to a concentration of 30 μM, had no such effects on the liver, as shown by the constant levels of label associated with this substance when [2-^{14}C]mevalonate was the cholesterol precursor (Fig. 2). It is possible that hepatic and non-hepatic cells respond differently in this respect to ketoconazole. The constancy of 24,25-oxidolanosterol under conditions in which HMG-CoA reductase activity declined (Fig. 5) lends support to the idea that the formation of this compound is not required for a suppression of reductase activity [17]. On the basis of serum sterol profiles in human subjects, it has also been suggested that high concentrations of the drug interfere with other post-lanosterol enzymes of cholesterol biosynthesis [18]. In the present work, the lack of any effect of ketoconazole on the formation of cholesterol from 4,4-dimethylcholesta-8,14-dienol, the immediate product of the demethylase reaction [26,28], suggested that, in liver subcellular fractions, at drug concentrations up to 10 μM, the post-14α-demethylase part of the pathway remained unperturbed. This also provided evidence that changes in reductase activity (Fig. 5) occur in the presence of an intact post-14α-demethylase section of the cholesterol pathway, and further highlights a specific link between lanosterol 14α-demethylation and HMG-CoA reductase activity.

Inhibition of lanosterol 14α-demethylase by relatively low (< 5–10 μM) concentrations of ketoconazole was accompanied by increases in the amounts of label associated with at least three oxygenated sterols. One of

these was identified as the C-32 aldehyde derivative of lanosterol or 24,25-dihydrolanosterol. This supports the work of Trzaskos and his colleagues [9,10,17] and, together with a recent report from Kandutsch's laboratory [20], provides strong circumstantial evidence for a natural role for this cholesterol precursor in the regulation of HMG-CoA reductase activity. One of the other, less highly labelled, polar sterols which accumulated in hepatocytes cultured with ketoconazole, had the chromatographic properties of 25-hydroxycholesterol, but its precise identity remains unknown. It did not correspond to lanost-8-ene-3β,32-diol. The remaining oxysterol fraction which accumulated under these conditions was more polar. Again, its identity is not known. How these substances arise, and whether they play a role in the suppression of HMG-CoA reductase activity (Fig. 5), are questions which remain to be resolved.

The other oxygenated intermediate of lanosterol demethylation, lanost-8-ene-3β,32-diol, did not accumulate in the presence of ketoconazole. Nevertheless, its use as a cholesterol precursor led to a ketoconazole-induced accumulation of its immediate product, 3β-hydroxylanost-8-en-32-al, in greater quantities than those which occurred when mevalonate and lanosterol were the cholesterol precursors. The accumulation of this substance implies that its further metabolism to 4,4-dimethylcholesta-8,14-dienol (structure IV, Scheme 1) is more sensitive to inhibition by ketoconazole than are the oxidative steps required for its formation. However, we have shown previously that when the ^{3}H-labelled C-32 aldehyde was the cholesterol precursor, although its metabolism was inhibited by ketoconazole, the extent of inhibition was less than that of the oxidative metabolism of lanosterol itself [28a]. The metabolism of the C-32 aldehyde was also insensitive to inhibition by CO, another inhibitor of cytochrome *P*-450 [14]. The kinetics of lanosterol demethylation are exceptionally complex, and this apparent paradox may be related to the increased interaction of the functionalized methyl group with the haem iron of oxidized cytochrome *P*-450 [29] and to the differential metabolism of enzyme-bound and non-enzyme-bound intermediates during cytochrome *P*-450-catalysed steroid demethylation [30]. These factors may help to explain the differential accumulation of the C-32 aldehyde when the C-32 alcohol, rather than lanosterol, was used as the cholesterol precursor (Fig. 6).

Apart from its effects on HMG-CoA reductase, ketoconazole induces changes in the expression of hepatic low-density lipoprotein receptors [18,31]. The molecular mechanism underlying this effect is unknown, but it seems reasonable to assume that steroidal oxidation(s) requiring cytochrome *P*-450 are involved. Thus oxysterols generated by this isoenzyme of cytochrome *P*-450 may be the signal by which information relating to cholesterol balance is transmitted through the cell via a specific oxysterol-binding protein [32–35]. If this is the case, then factors which regulate cytochrome *P*-450 activity may play an important role in determining the response of the cell to a shortage or excess of cholesterol.

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