Enzymatic Metabolism of Ergosterol by Cytochrome P450scc to Biologically Active 17α,24-Dihydroxyergosterol

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Summary

We demonstrate the metabolism of ergosterol by cytochrome P450scc in either a reconstituted system or isolated adrenal mitochondria. The major reaction product was identified as 17α,24-dihydroxyergosterol. Purified P450scc also generated hydroxyergosterol as a minor product, which is probably an intermediate in the synthesis of 17α,24-dihydroxyergosterol. In contrast to cholesterol and 7-dehydrocholesterol, cleavage of the ergosterol side chain was not observed. NMR analysis clearly located one hydroxyl group to C24, with evidence that the second hydroxyl group is at C17. 17α,24-Dihydroxyergosterol inhibited cell proliferation of HaCaT keratinocytes and melanoma cells. Thus, in comparison with cholesterol and 7-dehydrocholesterol, the 24-methyl group and the C22-C23 double bond of ergosterol prevent side chain cleavage by P450scc and change the enzyme's hydroxylase activity from C22 and C20, to C24 and C17, generating bioactive product.

Introduction

Cytochrome P450 side chain cleavage (P450scc), a product of the CYP11A1 locus, is a mitochondrial en-

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zyme, the main function of which has been purported to be the conversion of cholesterol to pregnenolone. There is a single active site on the cytochrome where successive hydroxylations of the cholesterol side chain occur at positions 22 and 20, followed by cleavage of the side chain to produce pregnenolone and isocapoic aldehyde [1, 2]. Most recently, 7-dehydrocholesterol has been uncovered as an additional substrate for P450scc, yielding 7-dehydropregnenolone as a final product [3, 4]. Aside from being a cholesterol precursor, 7-dehydrocholesterol is also a precursor for vitamin D3 through ultraviolet light B photolysis and temperature-dependent intramolecular rearrangement [5, 6].

Ergosterol, a 5,7-diene sterol, is synthesized by fungi and phytoplankton, but not in the animal kingdom [5]. Ergosterol serves as a major membrane sterol in fungi [7], and can serve as the precursor for the synthesis of vitamin D2 [5]. Ergosterol can act as a membrane antioxidant [8] and a modifier of the effect of cholesterol on human cell cycle progression [9]. Antitumor effects of ergosterol have been reported in cell culture [10] and in vivo in rats [11]. Anticancerogenic and antimutagenic properties of vitamin D2 (ergocalciferol) are well recognized [5, 12] and, because of their lower toxicity (minimal hypercalcemic effect), hydroxylated forms of vitamin D2 are considered as potential drugs for treatment of cancer patients [5, 13], including those with melanoma [14].

Ergosterol differs from 7-dehydrocholesterol in that its side chain has a C24-methyl group and C22-C23 double bond. P450scc has been shown to cleave the side chain of other plant sterols, including campesterol, which also has a C24-methyl group [15]. Because 7-dehydrocholesterol also serves as a substrate for P450scc [4] and has an identical ring system to ergosterol, we tested whether cytochrome P450scc can metabolize ergosterol.

Results

TLC and MS Characteristics of Ergosterol Metabolites of Purified P450scc

Incubation of both human and bovine P450scc with ergosterol in a phospholipid-vesicle-reconstituted system resulted in one major product and one minor product being observed upon analysis by TLC (Figure 1A). Both products were absent in the control incubations, where P450scc was omitted. The products, eluted from the TLC plate, had UV absorbance spectra identical to that for ergosterol (not shown). A small spot corresponding to pregnenolone (identity confirmed by mass spectra, data not shown) was observed in the test sample for bovine P450scc (Figure 1A, lane 3). This is produced from a small quantity of cholesterol, which copurifies with P450scc extracted from adrenal glands [16]. An electron impact mass spectrum of the major product identified it as dihydroxyergosterol (Figure 1C). It showed the molecular ion at m/z = 428, with fragment ions at m/z = 410 (428 – H_2O) and m/z = 395 (410 –
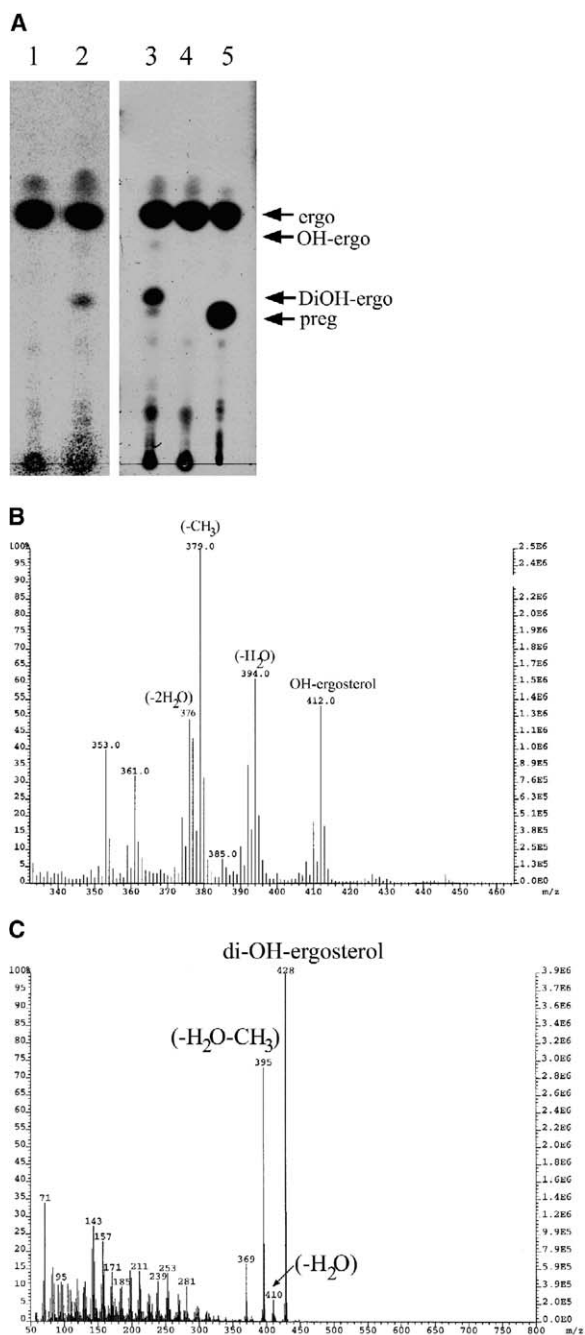
Figure 1. Analysis of Products of Ergosterol Metabolism in Vesicle-Reconstituted P450scc

Ergosterol, at a molar ratio to phospholipid of 0.2, was incubated for 1 hr with 0.75 μM human P450scc (lane 2) or 2 μM bovine P450scc (lane 3) in a phospholipid vesicle-reconstituted system, and the products analyzed by TLC (A). Lanes 1 and 4 are controls with all components present except P450scc. Lane 5 contains ergosterol (ergo) and pregnenolone (preg) standards. The arrows labeling OH-ergo and DiOH-ergo indicate the positions of the respective products of ergosterol metabolism, identified by EI mass spectrometry as hydroxyergosterol (B) and dihydroxyergosterol (C).

CH_3). In comparison, ergosterol has m/z = 396. These results show that the major action of P450scc on ergosterol is to add two hydroxyl groups. The minor product

was identified as hydroxyergosterol (Figure 1B). The molecular ion was at m/z = 412 with fragment ions m/z = 394 (412 − H_2O), m/z = 379 (394 − CH_3), and m/z = 361 (379 − H_2O). In contrast to cholesterol and 7-dehydrocholesterol, the incubation of ergosterol with P450scc did not yield products indicative of cleavage of the sterol side chain.

NMR Analysis of Dihydroxyergosterol
The NMR analysis of TLC-purified dihydroxyergosterol demonstrates that one OH group is located at C24 (Figure 2). Although the presences of impurities in both the methyl region (0.5–1.5 ppm) and the expected hydroxyl region (3.0–4.5 ppm) prevents a definitive analysis, the regions of the protons on the double bonds are relatively clean. Both the chemical shifts and coupling patterns of H6 and H7 are identical in ergosterol and this metabolite. However, H22 and H23 in the side chain are downshifted from 5.18 ppm and 5.22 ppm to 5.35 ppm. In addition, the correlation between H23 and H24 (chemical shift of 1.86 ppm in ergosterol) is missing, whereas that between H22 and H20 (chemical shift of 2.04 ppm in ergosterol) is still present in this dihydroxyl metabolite. This is a strong indication that H24 becomes hydroxylated in this metabolite. In addition, the doublet of 28-methyl protons in ergosterol (0.92 ppm for proton and 17.7 ppm for carbon) became a singlet and shifted downfield (1.3 ppm for proton and 24 ppm for carbon). This again clearly defines the hydroxylation at C24.

The presence of the impurities makes definitive assignment of the second hydroxyl location very difficult. Although further HPLC purification (retention time [RT] of 13.7) removed the impurities, the amount of purified ergosterol metabolite was insufficient. Nevertheless, the comparison of ergosterol and dihydroxyergosterol heteronuclear single quantum correlation (HSQC) spectra (Figure 2E) strongly indicates that the second hydroxyl group is located at C17. In Figure 2E, the two circled spots in the ergosterol HSQC spectrum (left) correspond to a correlation between 14-CH (1.89 ppm for proton and 54.7 ppm for carbon) and 17-CH (1.27 ppm for proton and 55.9 ppm for carbon). No such spots are visible in those regions for the dihydroxyl metabolite (right spectrum). The correlation peak of 14-CH in this metabolite shifted downfield to a new position (2.72 ppm for proton and 58.0 ppm for carbon), as indicated in the circle in the right spectrum, whereas the original 17-CH correlation disappeared. The shift of the 14-CH is caused by the formation of 17-OH in this metabolite. Hence, this dihydroxyl metabolite is most likely 17α,24-dihydroxylergosterol.

Based on the information presented in Figures 1 and 2, we propose that P450scc transforms ergosterol to 17α,24-dihydroxylergosterol, with 24-hydroxyergosterol serving as an intermediate of the metabolism (Figure 3).

The Rate of Ergosterol Metabolism by Purified P450scc
To obtain an estimate of the initial rate of ergosterol metabolism by P450scc, ergosterol, at a molar ratio to phospholipid of 0.2, was incubated with P450scc for 5
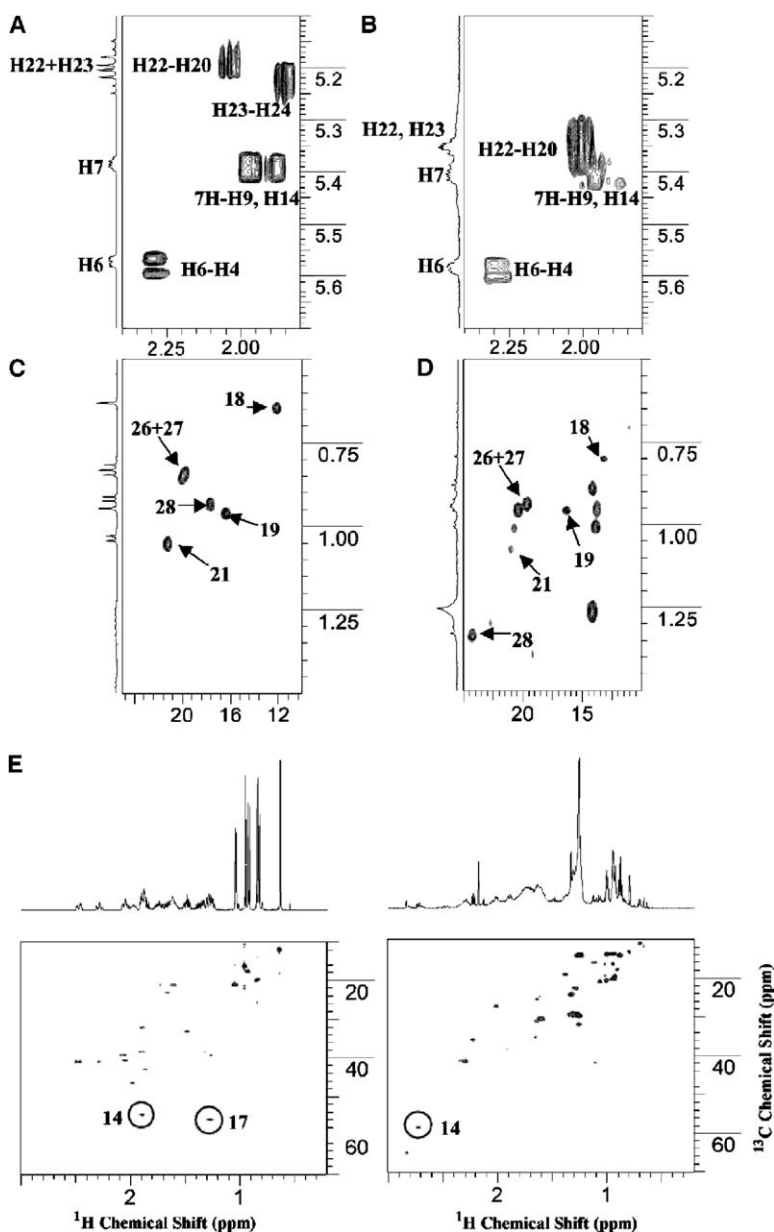
Figure 2. NMR Analysis of the Hydroxylation at Position 24-CH

Finger print regions in proton NMR spectra of (A) ergosterol proton correlation spectroscopy, (B) dihydroxyl metabolite, (C) ergosterol heteronuclear single quantum correlation (HSQC) methyls, and (D) dihydroxyl metabolite HSQC methyls. The corresponding proton 1D NMR spectra are shown as projections. The formation of 24-OH is clearly indicated by (1) the missing of H23-H24 correlation in spectrum B with concurrent downfield shift of H22 (5.18 ppm) and H23 (5.22 ppm) to 5.35 ppm; and (2) the transition of 28 doublet methyl at 0.92 ppm in spectrum C to a downfield singlet at 1.35 ppm in spectrum D. (E) Part of proton-carbon HSQC spectra of Ergosterol standard (left) and its dihydroxyl metabolite (right). The two circled spots in ergosterol HSQC spectrum (left) correspond to correlation between H14 (1.89 ppm) and C14 (54.7 ppm), and H17 (1.27 ppm) and C17 (55.9 ppm). No such spots are visible in those regions for ergosterol metabolite (right spectrum) due to 17-hydroxylation. The shift of HSQC correlation spot between H14 and C14 to the new position (2.72 ppm, 58.0 ppm) is caused by interaction of 14α hydrogen with the 17α-OH group.

min at 35°C. The dihydroxyergosterol was extracted, purified by TLC, and quantitated from its absorbance at 281 nm. This gave a rate of metabolism of 0.7 mol product/min/mol P450scc. Under similar conditions, cholesterol was converted to pregnenolone at a rate of 17.7 mol/min/mol P450scc [4].

The Effect of Ergosterol and Dihydroxyergosterol on the Spin State of Cytochrome P450scc

Cytochrome P450scc incorporated into phospholipid vesicles prepared from dioleoyl phosphatidylcholine and cardiolipin displays a typically low-spin spectrum of the substrate-free enzyme (Figure 4, spectrum 1),
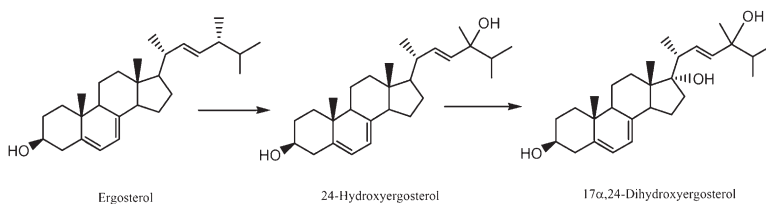

Figure 3. Proposed Sequence for the P450scc Catalyzed Transformation of Ergosterol with Structures of Expected Reaction Products
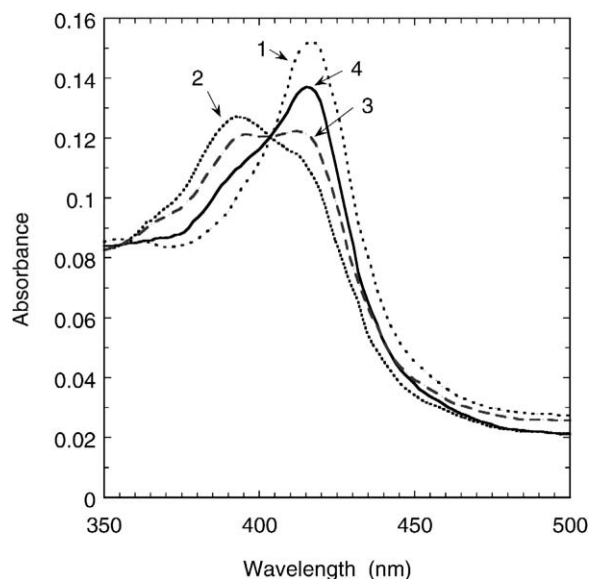
Figure 4. The Effects of Ergosterol and Dihydroxyergosterol on the Visible Absorbance of P450scc

P450scc was incorporated into phospholipid vesicles and spectra recorded against a reference cuvette containing all components except P450scc. (1) Vesicles containing no substrate; (2) vesicles containing 0.1 mol cholesterol/mol phospholipid; (3) vesicles containing both 0.1 mol cholesterol/mol phospholipid and 0.2 mol ergosterol/mol phospholipid; (4) vesicles containing both 0.1 mol cholesterol/mol phospholipid and 0.004 mol dihydroxyergosterol/mol phospholipid.
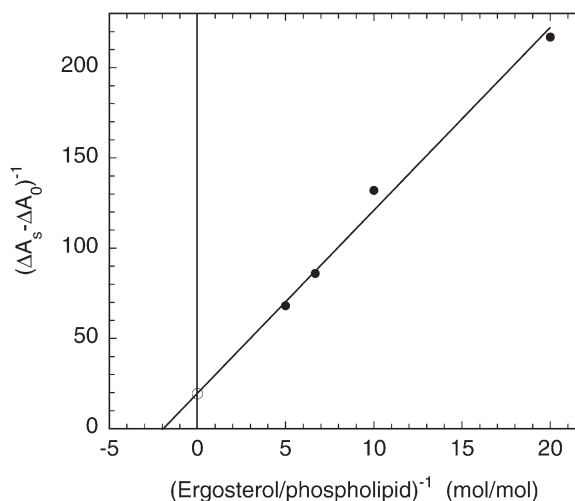

Figure 5. Double Reciprocal Plot Showing Ergosterol Binding to P450scc in Phospholipid Vesicles Containing Cholesterol

Vesicles contained 0.05 mol cholesterol/mol phospholipid and ergosterol at the indicated ratios. The open symbol on the Y axis represents the absorbance change expected for complete reversal of cholesterol-induced high-spin state back to the substrate-free low-spin state. ΔA_s, absorbance difference (416–392 nm) in the presence of ergosterol; ΔA_0, absorbance difference (416–392 nm) in the absence of ergosterol with 0.05 mol cholesterol/mol phospholipid present.

with maximum absorbance at 416 nm [17, 18]. The inclusion of ergosterol in the vesicles at a molar ratio to phospholipid of 0.2 did not alter the spectrum (data not shown). In contrast, the presence of cholesterol at a molar ratio to phospholipid of 0.1 caused a transition to the high-spin state, with maximum absorbance at 392 nm (Figure 4, spectrum 2). The magnitude of the change in the spin state provides an index of substrate binding, and we have previously used this to determine the K_d for cholesterol and hydroxycholesterol reaction intermediates, which bind competitively, in this system. [18–20]. Ergosterol induced a shift toward the low-spin state in the presence of cholesterol (Figure 4, spectrum 3). Thus, unlike cholesterol, ergosterol is a low-spin inducer of P450scc. 17α,24-Dihydroxyergosterol also proved to be a low-spin inducer (Figure 4, spectrum 4). This is similar to 22R-hydroxycholesterol, but different from 20α, 22R-dihydroxycholesterol, which is a high-spin inducer [18, 19].

The reversal of the high-spin shift induced by cholesterol was used to determine the strength of ergosterol binding to P450scc. In the presence of 0.05 mol cholesterol/mol phospholipid, an apparent K_d for ergosterol of 0.5 mol/mol phospholipid was obtained (Figure 5). Extrapolation of the graph to infinite ergosterol concentration (zero on the X axis of Figure 5) reveals that, as expected for competitive binding, ergosterol can completely reverse the spin-state shift induced by cholesterol. Analysis of the competitive binding (see Experimental Procedures), using the K_d for cholesterol

determined independently (0.033 mol cholesterol/mol phospholipid), indicated 0.2 mol/mol phospholipid as the true K_d for ergosterol. At a molar ratio to phospholipid of only 0.004, dihydroxyergosterol caused a substantial shift toward the low-spin state in the presence of 0.1 mol cholesterol/mol phospholipid. The limited availability of this product prevented us from determining its K_d, but from the concentration used and the magnitude of the absorbance change, its K_d is at least 30 times lower than that for cholesterol.

Ergosterol Metabolism by Adrenal Mitochondria
In agreement with the above results with purified P450scc, the RP-HPLC analysis of the reaction mixture resulting from incubation of adrenal mitochondria with ergosterol showed a single reaction product (metabolite 1) at RT 14.2 min (ergosterol RT is 49.5.min), which was absent in control samples (Figures 6A and 6B). The absorbance spectrum of this product was similar to that of ergosterol (Figures 6C and 6E) [4]. The mass spectrum of metabolite 1 yielded a molecular ion characteristic of the dihydroxyergosterol fragment with $[M+H]^+$ at m/z = 411 (429 – H_2O), and a fragment ion at m/z = 393 (429 – $2H_2O$) (Figure 6D). The mass spectrum of ergosterol standard (RT = 49.5 min), in addition to the expected ion m/z = 397 (minor product), also yielded the same ions at m/z = 393 (major), indicating that ergosterol (theoretical m/z = 397) had undergone desaturation during LC/MS. The ions $[M+H]^+$ at m/z = 375 and at m/z = 425 obtained during MS analysis of ergosterol (absent in the reaction product) represent methyl group (CH3) loss and methanol addition to desaturated ergosterol, respectively (Figure 6F). Thus, as
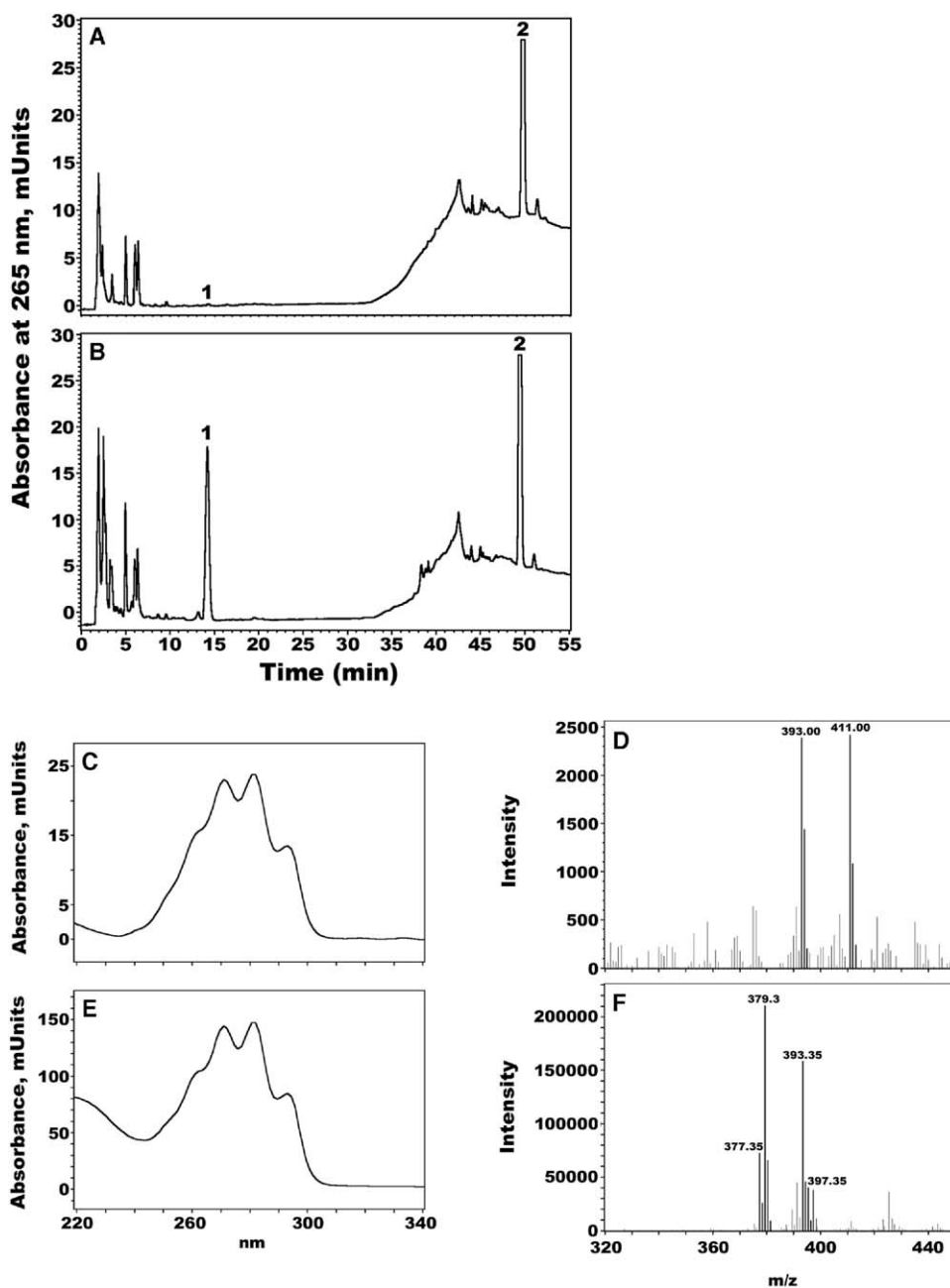
Figure 6. RP-HPLC Identification of a Product of Ergosterol Metabolism by Adrenal Mitochondria

(A) Control (incubation without NADPH and isocitrate).

(B) Experimental incubation (with NADPH and isocitrate). The HPLC elution profiles were monitored by absorbance at 265 nm; the number 1 marks as the metabolite and 2 the ergosterol.

(C) UV spectra of reaction product at RT 14.2 min.

(D) Mass spectra of the reaction product at RT 14.2 min.

(E) UV spectra of ergosterol (RT 49.5 min).

(F) Mass spectra of ergosterol.

for purified P450scc, the major product of ergosterol metabolism by P450scc in adrenal mitochondria is dihydroxyergosterol.

Dihydroxyergosterol Inhibits Proliferation of Human Skin Cells

Addition of 17α,24-dihydroxyergosterol (HPLC-purified) to the culture media inhibited DNA synthesis in human epidermal HaCaT keratinocytes, an effect that was absent in cultures treated with the ergosterol precursor (Figure 7). The growth inhibitory effect for 17α,24-dihydroxyergosterol was observed at both high concentrations (10^{-7} M and higher) and at low concentrations (10^{-9} M or lower), with a reproducible lack of effect at 10^{-8} M (Figures 7A and 7B). Similar results were further observed when 17α,24-dihydroxyergosterol was dis-
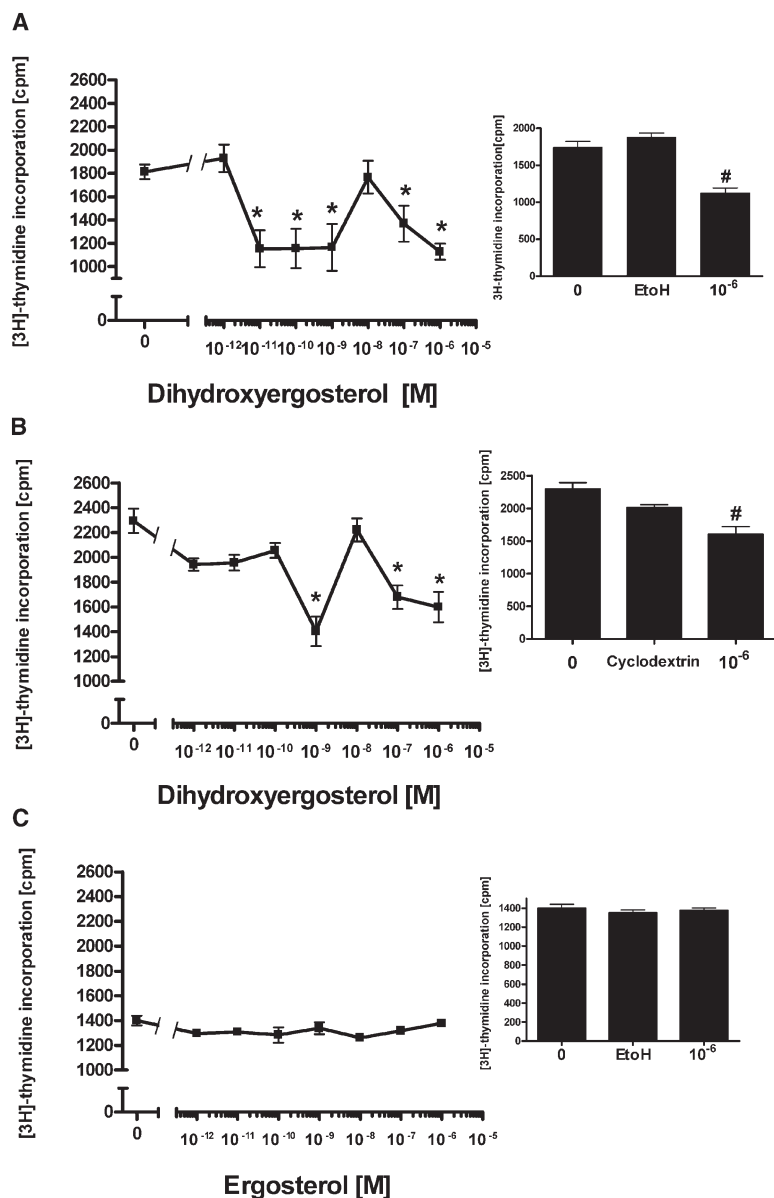
A

B

C

Figure 7. 17α,24-Dihydroxyergosterol but not Ergosterol Precursor Inhibits DNA Synthesis in Human Skin Cells

Cultures of HaCaT keratinocytes were synchronized and then incubated for 24 hr in media containing serial dilutions of 17α,24-dihydroxyergosterol dissolved in either EtOH (A) or cyclodextrin (B), or serial dilutions of ergosterol dissolved in EtOH (C). Data are presented as mean ± SEM (n = 8), analyzed with one-way analysis of variance with post hoc Dunnett's multiple comparison test (significance at $*p < 0.05$). Each experiment was performed twice. Insets in (A) and (B) show that solvents at 0.2% concentration did not modify DNA synthesis in control cultures, and that the inhibitory effect for dihydroxyergosterol at 10^{-6} M versus solvent is significant ($\#p < 0.05$).

solved in either ethanol or cyclodextrin. The hiatus in activity suggests that 17α,24-dihydroxyergosterol could act both directly and indirectly through a more potent metabolite, generated from 17α,24-dihydroxyergosterol during the incubation of HaCaT keratinocytes. We have also performed initial testing of melanoma cell line SKMEL-188 and found that both 17α,24-dihydroxyergosterol and ergosterol precursor inhibit DNA synthesis in a dose-dependent manner (data not shown).

Discussion

We were able to demonstrate that P450scc in an in vitro reconstituted system or in isolated mitochondria hydroxylates ergosterol to yield dihydroxyergosterol as the major product. There appears to be no subsequent metabolism of dihydroxyegrosterol by other steroido-

genic enzymes present in adrenal mitochondria under our experimental conditions. Generation of a minor product, hydroxyergosterol, was detected only by purified P450scc in a reconstituted system, indicating that it represents an intermediate in the transformation to the dihydroxyproduct (Figure 3). NMR analysis of dihydroxyergosterol demonstrated that one of the hydroxyl groups is at C24. Additional NMR analysis indicated that the second hydroxyl group was located at C17. We did not detect generation of 7-dehydropregnenolone from ergosterol in either the reconstituted system with purified P450scc or with rat adrenal mitochondria. This is in contrast to P450scc action on 7-dehydrocholesterol in a reconstituted system or in adrenal mitochondria assayed under identical conditions to those used for ergosterol, where 7-dehydropregnenolone is the sole detectable product [4]. This suggests that structural features of

ergosterol side chain (e.g., the presence of C22-C23 double bond) prevent its cleavage by P450scc, leading to a limited hydroxylation of the side chain at C24 and C17 but not at C20 or C22, which is in contrast to cholesterol and vitamin D3, in which P450scc hydroxylates at both of these positions [3, 21]. Furthermore, the ability to cleave the side chain of 24α-methylcholesterol (campesterol) by both human and bovine P450scc to produce pregnenolone [21] excludes the 24-methyl group as the major structural factor preventing the oxidative cleavage of the side chain of ergosterol by P450scc.

Binding of ergosterol and dihydroxyergosterol to P450scc induced a shift toward the low-spin state of the cytochrome. In contrast, cholesterol, 20α,22R-dihydroxycholesterol [18, 19], and 7-dehydrocholesterol (unpublished data) are high-spin inducers. Thus, the presence of the 24α-methyl group and the C22-C23 double bond of ergosterol alter the interaction with the haem iron to produce the low-spin state. Ergosterol binds to the active site of P450scc with an affinity only one sixth of that for cholesterol, which may in part explain its lower hydroxylation rate compared with that observed with an equivalent concentration of cholesterol. Our preliminary studies show that dihydroxyergosterol binding to P450scc is at least 30-fold tighter than that of cholesterol. This higher affinity relative to cholesterol is similar to the much tighter binding of 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol to P450scc, which helps ensure that the reaction goes to completion without the accumulation of intermediates [19, 22]. The tight binding of dihydroxyergosterol to P450scc likely results in competitive inhibition during catalysis, as this final product saturates the active site in preference to ergosterol. This might also contribute to the relatively slow rate of metabolism of ergosterol compared to cholesterol.

This surprising activity of mammalian P450scc toward ergosterol raises questions about its biological significance. Earlier studies reported that orally delivered ergosterol is absorbed by the alimentary tract (2%–5% of total intake) and enters the systemic circulation with highest accumulation and metabolism occurring in the liver, lung, spleen, and adrenals [23, 24]. More recent studies have confirmed the intestinal absorption of ergosterol and its subsequent accumulation and metabolism in the liver, intestine, skin, kidney, and blood [25]. It is noteworthy that the ergosterol metabolites detected in the skin were different from vitamin D2 [25]. In addition, cell culture studies clearly demonstrate that ergosterol is efficiently taken up by human cells (at 50% of the cholesterol uptake rate) [9]. Therefore, it can be assumed that transformation of ergosterol to dihydroxyergosterol will probably occur in organs expressing cytochrome P450scc, such as adrenals, gonads, placenta, brain [26], gastrointestinal tract [27], kidney [28], and skin [4]. Accordingly, we have demonstrated metabolism of ergosterol by mitochondria isolated from adrenals (Figure 6). Moreover, ergosterol antitumor effects have been noted in cell cultures [10] and in vivo [11].

Our cell culture studies demonstrate biological activity for 17α,24-dihydroxyergosterol, but not for ergosterol in terms of inhibition of proliferation of human immortalized epidermal keratinocytes. These results are consistent with reports of minimal or nondetectable effects for unmodified ergosterol on human cell cycling [9], whereas ergosterol metabolites have potent antiproliferative and anticancer effects on human and animal cells [10, 29–31]. It should also be noted that the product of B ring breakage in ergosterol (vitamin D2), promotes differentiation of keratinocytes in vivo, and protects them from UVR-induced photodamage when applied topically [12]. Concerning melanoma cells, we found that both ergosterol precursor and dihydroxyergosterol metabolite inhibit DNA sythesis, suggesting local metabolism of ergosterol; of note is the observation that melanoma cells do express P450scc [4].

In conclusion, we have demonstrated that ergosterol serves as a substrate for mammalian P450scc generating bioactive 17α,24-dihydroxyergosterol. This reaction proceeds in an in vitro reconstituted enzymatic system and in adrenal mitochondria, but is restricted to hydroxylations at positions C24 and C17 without detectable side chain cleavage. Thus, of the two vitamin D precursors that contain the 5,7-diene group in the B-ring, only 7-dehydrocholesterol (vitamin D3 precursor) can undergo side chain cleavage by P450scc, producing 7-dehydropregnenolone. Although the biological activity of the 7-dehydropregnenolone is unknown, 17α,24-dihydroxyergosterol inhibits proliferation of skin cells in culture.

Significance

The present work uncovers a function for the key enzyme in steroidogenesis, cytochrome P450scc, well known for its activity in cleaving the side chain of cholesterol in the synthesis of steroid hormones. It has been discovered that 7-dehydrocholesterol (precursor to vitamin D3) is an alternative substrate for cytochrome P450scc and has its side chain cleaved to produce 7-dehydropregnenolone (7-DHP). This finding appears to define a metabolic pathway that also provides an explanation for the increased concentrations of 7-DHP and its hydroxymetabolites in Smith Lemli Opitz Syndrome (7-dehydrocholesterol excess). Continuing our studies on vitamin D precursors, we now demonstrate that the plant sterol and precursor to vitamin D2, ergosterol, can also undergo hydroxylation by mammalian cytochrome P450scc in either a reconstituted system or isolated adrenal mitochondria. The major reaction product was identified as 17α,24-dihydroxyergosterol, with a minor product, monohydroxyergosterol, which most likely represents an intermediate in the synthesis of the dihydroxyderivative. In contrast to the action of P450scc on 7-dehydrocholesterol, cleavage of the side chain of ergosterol was not observed. Thus, the presence of the 24-methyl group and the C22-C23 double bond prevent side chain cleavage by P450scc, and shift the enzyme hydroxylase activity from C22 and C20 to C24 and C17. 17α,24-dihydroxyergosterol has biological activity (inhibition of proliferation), as demonstrated in human keratinocytes and melanoma cell lines. Ergosterol itself has been previously reported as being devoid of effects on human cell cycling, whereas ergosterol metabolites have significant

antiproliferative and anticancer properties. Dietary ergosterol is absorbed in the alimentary tract and also taken up by mammalian cultured cells. It accumulates in the adrenals and other organs, including skin, but without conversion to vitamin D2. Thus, when available to cells containing P450scc, ergosterol can be metabolized in vivo to generate newer bioactive products.

Experimental Procedures

Metabolism of Ergosterol by Reconstituted P450scc

P450scc and adrenodoxin reductase were purified from bovine adrenal mitochondria [18, 32]. Adrenodoxin and human P450scc were expressed in *Escherichia coli* (*E. coli*) and purified as previously reported [33]. Incubations of P450scc with ergosterol (Sigma) were carried out in artificial phospholipid vesicles prepared by sonication [34], as described for 7-dehydrocholesterol [4]. The incubation mixture was comprised 510 μM phospholipid vesicles (dioleoyl phosphatidylcholine plus 15 mol% cardiolipin), with a substrate to phospholipid molar ratio of 0.2, 50 μM NADPH, 2 mM glucose 6-phosphate, 2 U/ml glucose 6-phosphate dehydrogenase, 0.2 μM adrenodoxin reductase, 10 μM adrenodoxin, 2.0 μM cytochrome P450scc, and buffer, pH 7.4. The final volume was 2.0 ml for determining the rate of ergosterol metabolism and producing product for mass spectra, and 50 ml for preparing products for NMR. After incubation at 35°C for 1 hr (or other times as indicated in the Results section), the mixture was extracted twice with 2 volumes of methylene chloride, and the combined extracts dried under nitrogen at 35°C. The extract was analyzed by thin-layer chromatography on silica gel G with three developments in hexane:ethyl acetate (3:1, v/v), as previously described [4]. Products in selected lanes were visualized by charring. Corresponding products in other lanes were eluted from the silica gel as previously described [4] and the amount of products determined from their UV spectrum using an extinction coefficient of 9900 M^{-1} cm^{-1} at 281 nm, determined for the 5,7 diene structure of ergosterol.

Metabolism of Ergosterol by Adrenal Mitochondria

Adrenals were obtained from male Wistar rats aged 3 mo, terminated under anesthesia. The animals were housed at the vivarium of the Department of Biotesting of Bioorganic Chemistry Institute, Minsk, Belarus). The experiments were approved by the Belarus University Animal Care and Use Committee.

The adrenal mitochondrial fraction was prepared, and the washed mitochondrial fraction resuspended in 0.25 M sucrose and used for enzymatic reactions, as described previously [4]. Briefly, isolated mitochondria were preincubated (10 min at 37°C) with sterols ergosterol or 7-dehydrocholesterol (20 μM) dissolved in 45% 2-hydroxypropyl-cyclodextrin [4]. The reactions were started by adding NADPH (0.5 mM) and isocitrate (5 mM), and stopped after 90 min at 37°C, by adding ice-cold methylene chloride. The mixtures were re-extracted two more times, dried, dissolved in methanol, and analyzed on a HPLC mass spectrometer (LC-MS).

Measurement of Visible Absorbance Spectra for P450scc and Ergosterol Binding

Vesicles were prepared from phosphatidylcholine and bovine heart cardiolipin using a bath-type sonicator in buffer comprising 20 mM Hepes (pH 7.4), 100 mM NaCl, 0.1 mM dithiothreitol, and 0.1 mM EDTA, as described previously [34]. The cardiolipin content was 15 mol% of the total phospholipid. Ergosterol, dihydroxyergosterol and/or cholesterol were included in the vesicles as required (see Results). Purified P450scc (1.0 μM) was incorporated into the vesicles (400 μM phospholipid) by incubation at room temperature for 30 min in a final volume of 0.7 ml [34]. Spectra were recorded between 350 and 500 nm against a reference cuvette containing all components except P450scc. The K_d for cholesterol was determined by titrating the absorbance change between 316 and 412 nm with cholesterol, as previously described [19]. The K_d for ergosterol was determined by its ability to reverse the absorbance change induced by cholesterol using competitive binding analysis

with the equation: $K_d = K_{d, app}/(1 + [I]/K_I)$, where $K_{d, app}$ is the apparent K_d for ergosterol in the presence of cholesterol, I is the cholesterol concentration, and K_I is the K_d for cholesterol. This method has been used previously to determine K_d values for 20α-hydroxycholesterol and 22R-hydroxycholesterol binding to P450scc [19].

Mass Spectrometry

Products of ergosterol metabolism by purified P450scc were eluted from TLC plates, dissolved in ethanol, and electron impact (EI) mass spectra were recorded with a Micromass VG Autospec mass spectrometer operating at 70 eV, with scanning from 800 to 50 at 1 sec/decade.

The products of incubation of ergosterol with mitochondria were analyzed on an LCMS-QP8000α (Shimadzu, Kyoto, Japan) equipped with a Restec Allure C18 column (150 × 4.6 mm; 5 μm particle size; and 60 Å pore size), UV/VIS photodiode array detector (SPD-M10Avp), and quadrupole mass spectrometer. The LC-MS workstation Class-8000 software was used for system control and data acquisition (Shimadzu, Japan). Elution was carried out at 40°C with a flow rate of 0.75 ml/min. The mobile phases consisted of 85% methanol and 0.1% acetic acid from 0 to 25 min, followed by a linear gradient to 100% methanol and 0.1% acetic acid from 25 to 35 min; and 100% methanol and 0.1% acetic acid from 35 to 55 min. The MS operated in atmospheric pressure chemical ionization positive-ion mode, and nitrogen was used as the nebulizing gas. The MS parameters were as follows: the nebulizer gas flow rate was 2.5 l/min, probe high voltage was 3.5 kV, probe temperature was 300°C, and the curved desolvation line heater temperature was 250°C. Analyses were carried out in scan mode from m/z 320 to 450 or, in SIM mode, at the expected m/z of the standards.

Nuclear Magnetic Resonance

TLC-purified (see above) dihydroxyergosterol (the mass of the compound was confirmed by MS) was dissolved in CDCl$_3$ (Cambridge Isotope Laboratories, Inc., Andover, MA). and NMR spectra were acquired using a Varian Inova-500 M NMR equipped with a 4 mm gHX Nanoprobe (Varian NMR, Inc., Palo Alto, CA). The total volume in the NMR rotor was 40 μl, and all spectra were acquired at a temperature of 294 K, with a spinning rate of 2500 Hz. Proton 1D NMR, proton correlation spectroscopy and proton-carbon correlation spectroscopy (HSQC) were acquired and processed with standard parameters. Possible positions of the hydroxyl groups in the metabolite were analyzed by comparing the acquired spectra with those of parent ergosterol.

Because of the impurities in TLC-purified dihydroxyergosterol (impairing assignment of one of the hydroxyl groups; see below), it was purified by RP-HPLC through Allure C18 column (150 × 4.6 mm; 3 μm particle size; Restek Corporation, Bellefonte, CA), following the procedure described for LC-MS, and then analyzed by NMR (see above).

Cell Proliferation Assays

The assays followed methodology routinely used in our laboratory [35]. Briefly, HaCaT keratinocytes and SKMEL-188 melanoma cells were respectively cultured in DMEM or Ham's F10 medium, supplemented with 5% FBS and 1% antibiotic solution (GIBCO). To measure DNA synthesis, the cells were seeded into 96-well plates (5,000 per well). After 6 hr, the medium was replaced with serum-free Ham's F10 medium to synchronize the cells in cell cycle [36]. After 12 hr, the medium was changed to Ham's F10, containing 5% FBS plus graded dilutions of dihydroxyergosterol. In control cells, dihydroergosterol was replaced with solvent ethanol (EtOH) or α-cyclodextrin (0.2% solvent corresponding to the same concentration as in dihydroxyergosterol at 10^{-6} M). Twelve hours later, it was replaced with fresh medium containing serial dilutions of dihydroxyergosterol and [3H]-thymidine (1 μCi/ml) for 12 hr incubation. The media were then discarded, cells detached with trypsin, harvested on fiber glass filter, and radioactivity (proportional to methyl-[³H]thymidine incorporated into DNA) counted with a Packard direct β counter (Packard, Meriden, CA).

Data were analyzed with one-way analysis of variance with post hoc Dunnett's Multiple Comparison Test using Prism 4.00 (Graph-

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