Human Cytochrome P450_{scc} (CYP11A1) Catalyzes Epoxide Formation with Ergosterol^S

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ABSTRACT:

Cytochrome P450_{scc} (P450_{scc}) catalyzes the cleavage of the side chain of both cholesterol and the vitamin D₃ precursor, 7-dehydrocholesterol. The aim of this study was to test the ability of human P450_{scc} to metabolize ergosterol, the vitamin D₂ precursor, and define the structure of the major products. P450_{scc} incorporated into the bilayer of phospholipid vesicles converted ergosterol to two major and four minor products with a k_{cat} of 53 mol · min⁻¹ · mol P450_{scc}⁻¹ and a K_m of 0.18 mol ergosterol/mol phospholipid, similar to the values observed for cholesterol metabolism. The reaction of ergosterol with P450_{scc} was scaled up to make enough of the two major products for structural analysis. From mass spectrometry, NMR, and comparison of the NMR data to that for similar molecules, we determined the structures of the two major products as 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol and 22-keto-23-hydroxy-22,23-dihydroergosterol. Molecular modeling and

nuclear Overhauser effect (or enhancement) spectroscopy spectra analysis helped to establish the configurations at C20, C22, and C23 and determine the final structures of major products as 22*R*,23*S*-epoxyergosta-5,7-diene-3 β ,20 α -diol and 3 β ,23*S*-dihydroxyergosta-5,7-dien-22-one. It is likely that the formation of the second product is through a 22,23-epoxy (oxirane) intermediate followed by C22 hydroxylation with the formation of strained 22-hydroxy-22,23-epoxide (oxiranol), which is immediately transformed to the more stable α -hydroxyketone. Molecular modeling of ergosterol into the P450_{scc} crystal structure positioned the ergosterol side chain consistent with formation of the above products. Thus, we have shown that P450_{scc} efficiently catalyzes epoxide formation with ergosterol giving rise to novel epoxy, hydroxy, and keto derivatives, without causing cleavage of the side chain.

Introduction

Ergosterol, a 5,7-diene sterol, is synthesized by fungi and phytoplankton but not in the animal kingdom (Holick, 2003). Ergosterol is a major membrane sterol in fungi (Bracher, 2003) and can serve as the precursor for the synthesis of vitamin D₂ via UV irradiation (Holick, 2003). It differs from 7-dehydrocholesterol, pro-vitamin D₃, in that its side chain has a C24-methyl group and C22=C23 double bond. Little is known about the metabolism of ergosterol in humans. Ergosterol taken up by the gut may act as a membrane antioxidant (Wiseman,

1993). Ergosterol can modify the effect of cholesterol on human cell cycle progression (Suárez et al., 2002) and has antitumor effects in cell culture (Yazawa et al., 2000) and in vivo in rats (Mitani et al., 2004).

Cytochrome P450_{scc} (P450_{scc}) catalyzes the first enzymatic step in steroid hormone synthesis, the cleavage of the cholesterol side chain after hydroxylations at C22 and C20 (Tuckey, 2005). P450_{scc} can also cleave the side chain of 7-dehydrocholesterol in a similar manner to the reaction on cholesterol both in vitro (Guryev et al., 2003; Slominski et al., 2004) and ex vivo in adrenal glands (Slominski et al., 2009). Furthermore, it can hydroxylate both vitamins D₂ and D₃ (Slominski et al., 2005a, 2006; Tuckey et al., 2008a,b; Nguyen et al., 2009).

We have previously reported that bovine P450_{scc} can hydroxylate ergosterol producing 17 α ,24-dihydroxyergosterol as the major product with no evidence for cleavage of the side chain (Slominski et al., 2005b). Having the same side chain as ergosterol, vitamin D₂ is metabolized by bovine P450_{scc} to 20-hydroxyvitamin D₂, 17,20-

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ABBREVIATIONS: P450_{scc}, cytochrome P450_{scc}; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NOE, nuclear Overhauser effect.

dihydroxyvitamin D₂, and 17,20,24-trihydroxyvitamin D₂ (Nguyen et al., 2009). 20-Hydroxyvitamin D₂ inhibits proliferation and stimulates differentiation of keratinocytes, melanocytes, and leukemia cells in a similar fashion to 1 α ,25-dihydroxyvitamin D₃, but unlike this hormone, it lacks calcemic activity in rats and, therefore, has therapeutic potential (Slominski et al., 2011). Opening of the B-ring of the steroid is not required for biological activity because the 17 α ,24-dihydroxy-ergosterol produced by bovine P450scc can inhibit the proliferation of both human keratinocytes and melanocytes (Slominski et al., 2005b). Steroids derived from 7-dehydrocholesterol (the vitamin D₃ precursor), observed in Smith-Lemli-Opitz syndrome (Shackleton et al., 2002), have also been shown to exhibit biological activity on skin cells without B-ring opening (Slominski et al., 2009, 2010). The metabolism of ergosterol by cytochrome P450 enzymes may, therefore, result in the production of derivatives of both physiological and pharmacological importance.

In the present study, we have examined the metabolism of ergosterol by human P450scc. Because P450scc is expressed in the gut where it plays a role in local corticosteroid production (Fernandez-Marcos et al., 2011), it could play a role in first-pass metabolism of ergosterol before it reaches the liver for excretion. Furthermore, P450scc is expressed in several other extra-adrenal and extragonadal tissues including skin, where it serves as a starting point for local steroids synthesis (Slominski et al., 2004, 2007), metabolizes 7-dehydrocholesterol (animal equivalent of plant ergosterol) (Slominski et al., 2009), or potentially may play a role in nonclassic vitamin D metabolism (Slominski et al., 2005a, 2011). Thus, when topically applied, ergosterol may serve as a substrate for P450scc with potential implications in therapy of skin hyperproliferative or inflammatory disorders. In fact previous experiments indicate that dihydroxyergosterol metabolites of bovine P450scc can inhibit proliferation of immortalized human epidermal keratinocytes (Slominski et al., 2005b).

In this manuscript, we show that human P450scc efficiently metabolizes ergosterol with production of novel derivatives containing a C22-C23 oxirane ring or an α -hydroxyketone in the side chain.

Materials and Methods

Materials. Cyclodextrin (2-hydroxypropyl- β -cyclodextrin), ergosterol, dioleoyl phosphatidylcholine, bovine heart cardiolipin, and NADPH were from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). The pGro7 plasmid was from Takara Bio Inc. (Shiga, Japan), and Alugram Sil G silica gel plates were from Macherey-Nagel, Inc. (Easton, PA).

Preparation of Enzymes. Human adrenodoxin and adrenodoxin reductase were expressed in *Escherichia coli* and were purified as described previously (Woods et al., 1998; Tuckey et al., 2011). Human P450scc was expressed similarly to our previous report (Woods et al., 1998) but with pGro7 plasmid present (Tang et al., 2010), which increased the P450scc expression level from 35 nM to approximately 800 nM. The P450scc from a 1-liter culture was extracted from the bacterial membrane fraction as described previously (Woods et al., 1998) except that 1% sodium cholate (without Emulgen 911) was used. The extract was centrifuged at 107,000g for 60 min to remove insoluble debris, and the supernatant was applied to a 6 $\times$ 2.5 cm hydroxyapatite column equilibrated with buffer comprising 20 mM potassium phosphate (pH 7.4), 0.1 mM dithiothreitol, 0.1 mM EDTA and 20% glycerol. The column was washed with 100 ml of the same buffer containing 0.25% sodium cholate then the P450scc was eluted by including 500 mM potassium phosphate in the wash buffer. The P450scc was dialyzed against 1 L 20 mM potassium phosphate (pH 7.4), 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.05% cholate, and 20% glycerol, concentrated to 30 μ M and stored at -80°C until use. This scheme produced a large amount of partially pure enzyme with high activity ($k_{\text{cat}} = 56 \text{ mol pregnenolone} \cdot \text{min}^{-1} \cdot \text{mol P450scc}^{-1}$ with cholesterol as substrate) and was used for the large-scale synthesis of ergosterol derivatives. More highly purified P450scc was used for some small-scale incubations and was purified by phenyl Sepharose and DEAE-Sepharcel (both from GE

Healthcare, Rydalmere, NSW, Australia) chromatography as described previously (Woods et al., 1998). Both preparations gave similar results for ergosterol metabolism.

Large-Scale Incubations of Ergosterol with Cytochrome P450scc and Purification of Major Products. A stock solution of ergosterol was prepared by dissolving it in 45% cyclodextrin to a final concentration of 4 mM and stirring in the dark for 3 days at room temperature (Tuckey et al., 2008b). Incubations (12.5 ml) were performed in buffer comprising 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 2 μ M human P450scc, 10 μ M adrenodoxin, 0.3 μ M adrenodoxin reductase, 2 mM glucose 6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase, and 50 μ M NADPH. A stock solution of ergosterol in cyclodextrin (0.37 ml) was added to the incubation mixture to give a final ergosterol concentration of 120 μ M and a cyclodextrin concentration of 1.3%. Samples were preincubated for 8 min, reactions were started by the addition of NADPH, and incubations were performed for 3 h at 37 $^{\circ}\text{C}$ with shaking. Reactions were stopped by the addition of 20 ml of ice-cold dichloromethane, and products were extracted as described previously (Tuckey et al., 2011). Extracts combined from two incubations were applied as a band to a 20 cm $\times$ 20 cm $\times$ 0.2 mm Silica Gel G plate with chloroform. Ergosterol standards and 1% of the extract were run separately as spots on either side of the plate, using a similar procedure to that described previously (Slominski et al., 2005). Thin-layer chromatography (TLC) plates were developed 3 times in hexane/ethyl acetate (3:1 v/v), and areas of the plate containing standard ergosterol and the 1% of the reaction mixture were removed, sprayed with a solution of 2 mM FeSO₄ containing 5% concentrated sulfuric acid and 5% glacial acetic acid, and then charred by heating to reveal the position of standard ergosterol ($R_f = 0.48$) and the major product (product C and D not separated, $R_f = 0.31$). These strips were then aligned with the remainder of the plate, and the positions of the major product were marked. This area was removed from the unstained section of the plate, and products were eluted from the silica gel with three 15-ml aliquots of CHCl₃/CH₃OH (1:1, v/v). The solvent was removed under nitrogen at 30 $^{\circ}\text{C}$, and samples were dissolved in 4 ml of methanol and then filtered through a 0.1- μ M filter to remove remaining silica particles.

Further purification of the products was performed using a PerkinElmer high-performance liquid chromatography (HPLC) system equipped with a UV monitor set at 280 nm (PerkinElmer Life and Analytical Sciences, Waltham, MA). The filtered extract from the TLC plate containing the major products (C and D) was chromatographed on a preparative C18 column (Brownlee Aquapore, 25 cm $\times$ 10 mm, particle size 20 μ m; PerkinElmer Life and Analytical Sciences) using an isocratic mobile phase of 83% methanol in water at a flow rate of 1.5 ml/min. This removed minor contaminants but did not separate products C and D. The major peak was collected and rechromatographed on a C18 column (Brownlee Aquapore 22 cm $\times$ 4.6 mm, particle size 7 μ m) using an isocratic mobile phase of 53% acetonitrile in water at a flow rate of 0.5 ml/min, which separated products C and D (see *Results*). The yield of products was determined spectrophotometrically at 282 nm using an extinction coefficient of $9900 \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$ determined for ergosterol (Slominski et al., 2005b).

Small-Scale Incubations of Ergosterol with Cytochrome P450scc. Vesicles were prepared from dioleoyl phosphatidylcholine and bovine heart cardiolipin in the ratio 85:15 (mol/mol). Ergosterol or cholesterol was added to the phospholipid as required (see *Results*). Buffer comprising 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 mM dithiothreitol, and 0.1 mM EDTA was added to 1.25 μ mol of phospholipid, and the mixture (0.5 ml) was sonicated for 10 min in a bath-type sonicator (Lambeth et al., 1982). P450scc was added to the vesicles, and incubations were performed at 37 $^{\circ}\text{C}$ in the presence of 15 μ M adrenodoxin and 0.5 μ M adrenodoxin reductase for 2 min (kinetic experiments) or up to 1 h (time courses), as described in detail previously (Tuckey et al., 2008b). After extraction with dichloromethane, samples were applied to a Grace Alltima (Grace Davidson Discovery Science, Baulkham Hills, NSW, Australia) C18, 25 cm $\times$ 4.6 mm column and were eluted with a gradient of 45 to 100% acetonitrile in water for 15 min at 1 ml/min, 100% acetonitrile for 15 min at 1 ml/min, followed by 100% methanol for 20 min at 1.5 ml/min. Pregnenolone formation from cholesterol was determined by radioimmunoassay (Tuckey and Cameron, 1993). Calculation of kinetic constants was also performed as described previously with the Michaelis-Menten equation being fitted to the data using KaleidaGraph 4.1 (Synergy Software, Reading, PA) (Tuckey et al., 2008b).

NMR Spectroscopy. NMR measurements were performed using an inverse triple-resonance 3-mm probe on an Agilent Inova 500-MHz spectrometer running VNMRJ 2.2D (Agilent Technologies, Santa Clara, CA) or using a 1.7-mm cryogenic probe on a Bruker 600 MHz spectrometer running Topspin 3.0 (Bruker Daltonics, Billerica, MA). Samples were dissolved in CD₃OD and were transferred to a 3-mm Shigemi NMR tube (Shigemi Inc., Allison Park, PA) or a 1.7-mm NMR tube. Temperature was regulated at 22°C and was controlled with an accuracy of $\pm 0.1^\circ\text{C}$. Chemical shifts were referenced to residual solvent peaks for CD₃OD (3.31 ppm for proton and 49.15 ppm for carbon). Standard two-dimensional NMR experiments [¹H-¹H correlation spectroscopy (COSY), ¹H-¹H total correlation spectroscopy (TOCSY, mixing time 80 ms), ¹H-¹H nuclear Overhauser effect spectroscopy (NOESY, mixing time 500 ms), ¹H-¹³C heteronuclear single quantum correlation spectroscopy (HSQC), and ¹H-¹³C heteronuclear multiple-bond correlation spectroscopy (HMBC)], were acquired to fully elucidate the structures of the metabolites. All data were transferred to an offline personal computer data station and were processed using ACD software version 12.0 (Advanced Chemistry Development, Toronto, ON, Canada), with zero-filling in the direct dimension and linear prediction in the indirect dimension.

Molecular Modeling. We selected the crystal structure of human P450_{ssc} in complex with 20,22-dihydroxycholesterol (Protein Data Bank code 3NA0) for modeling approaches (Strushkevich et al., 2011). We used Schrodinger Molecular Modeling Suite 2011 (Schrödinger Inc., Portland, OR) for these docking studies using similar procedures to those described previously (Chen et al., 2010, 2011). Briefly, molecules were built and prepared using the Ligprep module, and they were docked into the active site of P450_{ssc} using the Glide module in Schrodinger Suite. The best docking complexes were subjected to restricted molecular dynamics to release any strains by using the MacroModel module with the OPLS-2005 force field that is supplied with the software. The ligand and its surrounding residues within 15 Å were allowed to move freely, whereas residues outside the 15-Å radius were kept rigid.

Other Procedures. The concentration of P450_{ssc} was determined from the CO-reduced minus reduced difference spectrum using an extinction coefficient of $91,000 \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$ for the absorbance difference between 450 and 490 nm (Omura and Sato, 1964). Mass spectra were acquired in a Bruker Esquire liquid chromatography/mass spectrometry system (Bruker Daltonics) using the ionization source of electrospray ionization. Data were collected using Bruker Esquire Control software version 4.0, transferred to an offline personal computer data station, and processed by ACD mass processor. Fourier transform-infrared spectra were acquired in a PerkinElmer Spectrum-100 instrument equipped with a diamond Attenuated Total Reflectance reflection top plate (PerkinElmer Life and Analytical Sciences).

Results

Metabolism of Ergosterol by Human Cytochrome P450_{ssc}. To examine the ability of human P450_{ssc} to act on ergosterol, the

FIG. 1. Chromatogram showing the metabolites produced by P450_{ssc} action on ergosterol. Phospholipid vesicles containing 0.2 mol ergosterol/mol phospholipid were incubated with 2.0 μM human P450_{ssc} for 1 h, and products were extracted and analyzed by reverse-phase HPLC. The arrow on the x-axis indicates the transition to 100% methanol. No products were observed in control incubations where adrenodoxin was omitted (data not shown).

FIG. 2. Time course for ergosterol metabolism by P450_{ssc} in phospholipid vesicles. Incubations were performed, and products were analyzed as in Fig. 1. A, ergosterol depletion and formation of major products. B, minor products.

P450_{ssc} and ergosterol were incorporated into phospholipid vesicles made from dioleoylphosphatidylcholine and cardiolipin as a model for the inner mitochondrial membrane, where P450_{ssc} is located (Tuckey et al., 1985, 2005; Headlam et al., 2003). This system has been employed to study the kinetics of P450_{ssc} action on numerous substrates including cholesterol and its hydroxy derivatives (Lambeth et al., 1982; Tuckey and Stevenson, 1985; Tuckey, 2005), vitamin D₃ (Tuckey et al., 2008b, 2011) and vitamin D₂ (Nguyen et al., 2009), as well as for metabolism of vitamin D derivatives by CYP27B1 (Tang et al., 2010). Human P450_{ssc} in vesicles converted ergosterol into two major and four minor products, as indicated in the HPLC chromatogram (Fig. 1) and time course (Fig. 2). None of the products had the retention time of 7-dehydropregnenolone, the expected product if cleavage of the side chain were to occur by a mechanism similar to that for cholesterol or 7-dehydrocholesterol. There was little change to major products C and D from 20 to 60 min of incubation, but there was an increase in product F and a decrease in product A. Product E displayed a lag in its production, suggesting that it is a secondary metabolite that is produced only after the accumulation of one of the other metabolites. By 60 min of incubation, over 50% of the ergosterol was consumed.

Kinetics of Ergosterol Metabolism by Human P450_{ssc}. To enable us to compare the rates of metabolism of ergosterol and cholesterol by human P450_{ssc}, kinetic constants were compared using the phospholipid-vesicles reconstituted system under initial rate conditions, where ergosterol consumption was linear with time (2-min incubation) and less than 10% of the substrate was consumed. The kinetics of ergosterol and cholesterol metabolism by human P450_{ssc} were very similar. Ergosterol was metabolized with a k_{cat} of $53 \pm 14 \text{ mol} \cdot \text{min}^{-1} \cdot \text{mol P450}_{\text{ssc}}^{-1}$ and a K_m of $0.18 \pm 0.10 \text{ mol ergosterol/mol phospholipid}$ (data are mean $\pm$ S.E. from the hyperbolic curve fit). Cholesterol was converted to pregnenolone with a k_{cat} of 56 ± 8

TABLE 1

*NMR chemical shift assignments for ergosterol and its derivatives by analysis of their two-dimensional NMR*Carbon chemical shifts were obtained from detected signals in HSQC and HMBC experiments (solvent: CD₃OD).

Atom	Ergosterol		22 <i>R</i> ,23 <i>S</i> -Epoxyergosta-5,7-diene-3β,20α-diol		3β,23 <i>S</i> -Dihydroxyergosta-5,7-dien-22-one	
	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C
1	1.31/1.91	38.3	1.29/1.91	39.3	1.30/1.91	39.4
2	1.82/1.44	31.4	1.84/1.47	32.4	1.48/1.83	32.4
3	3.50	69.7	3.51	70.7	3.51	70.8
4	2.40, 2.25	40.1	2.41/2.26	41.2	2.41/2.23	41.3
5	NA	140.0	NA	141.3	NA	141.3
6	5.53	119.5	5.54	120.3	5.55	121.3
7	5.37	116.4	5.39	117.6	5.39	118.7
8	NA	140.9	NA	141.7	NA	ND
9	1.96	46.3	1.96	47.3	1.98	47.3
10	NA	37.0	NA	38.7	NA	37.6
11	1.63/1.76	20.8	1.63/1.76	21.8	1.65/1.77	21.8
12	1.27, 2.09	39.1	1.36/2.20	40.3	1.38/2.10	40.1
13	NA	42.6	NA	44.1	NA	44.0
14	1.93	54.5	1.92	55.3	1.92	54.5
15	1.37/1.69	22.8	1.47/1.79	23.5	1.46/1.73	24.0
16	1.29/1.76	29.5	1.47/1.78	23.4	1.30/1.73	28.0
17	1.29	55.8	1.74	59.8	1.72	54.5
18	0.66	11.2	0.80	13.6	0.70	12.0
19	0.94	15.4	0.95	16.4	0.95	16.4
20	2.06	40.7	NA	72.7	2.84	45.2
21	1.05	20.3	1.29	23.4	1.12	16.6
22	5.22	135.8	2.86	66.2	N/A	218.4
23	5.22	131.9	2.75	59.3	4.31	80.3
24	1.86	43.1	1.11	42.8	1.68	42.0
25	1.47	33.2	1.68	32.1	1.69	31.8
26, 27	0.84/0.87	18.8/19.1	0.96/0.98	19.6/20.6	0.99/1.04	20.5/21.0
28	0.94	16.9	0.98	13.6	0.75	11.2

NA, not applicable (ternary carbons) because chemical shifts could not be unambiguously determined because of overlapping or weak signals at this position; ND, not determined.

mol · min⁻¹ · mol P450scc⁻¹ and a K_m of 0.16 ± 0.04 mol cholesterol/mol phospholipid.

Large-Scale Production of Ergosterol Metabolites. To permit production of sufficient quantity of the major products so that their structure could

be determined by NMR, four 12.5-ml incubations of human P450scc with ergosterol were performed. The cyclodextrin system was chosen because of its ease of use and greater conversion of substrate to products compared to phospholipid vesicles. After extraction and purification by TLC and HPLC

FIG. 3. NMR spectra of 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol [22*R*,23*S*-epoxyergosta-5,7-diene-3β,20α-diol]. A, one-dimensional proton. B, ¹H-¹³C HSQC. C, ¹H-¹³C HMBC. Full spectra are shown in supplemental figures.

FIG. 4. The possible two isomers for product C. Experimental data suggest the structure of product C is consistent with that of the 22*R*,23*S*-isomer.

(see *Materials and Methods*), 270 μ g of product C and 90 μ g of product D were obtained for NMR and mass spectral analysis.

Determination of the Structure of Product C as 20-Hydroxy-22,23-epoxy-22,23-dihydroergosterol. The molecular weight for this metabolite is 428 based on the observed molecular ion of 451 $[M + Na]^+$ (Supplemental Fig. 1A), indicating that two oxygen atoms are added to the parent ergosterol (molecular weight of 396.3). This is consistent with its increased polarity as revealed by HPLC analysis in comparison to ergosterol (Fig. 1). By thorough analysis of its NMR spectra, we determined the oxygenation sites are C20, C22, and C23, with an epoxide ring formed between C22 and C23, as described below.

Compared with the proton chemical shift in ergosterol (Table 1), the methyl protons at C21 are a singlet ($\delta = 1.29$ ppm; Fig. 3A; Table 1) in this metabolite instead of a doublet in ergosterol ($\delta = 1.05$ ppm). The chemical shift for C20 was downfield to 72.7 ppm in this metabolite, compared with 40.7 ppm in ergosterol (Table 1). The loss of scalar coupling to the proton at C20 and the downfield shifts clearly indicate the hydroxylation is at C20. The characteristic proton chemical shift for the 21-methyl group suggests a 20 α -hydroxyl configuration (according to Fischer projection), consistent with many reported 20-hydroxylation metabolites (Mijares et al., 1967; Corey et al., 1991; Li et al., 2010). All other methyl protons are intact.

Two other major changes in the proton NMR spectra of this metabolite are the loss of the C22=C23 double bond and the significant upfield shifting for both protons (5.22 ppm to 2.75 and 2.86 ppm) and carbons (135.8 to 66.2 ppm and 131.9 to 59.3 ppm) at C22 and C23 compared with those in ergosterol (Table 1; Fig. 3B, HSQC inset). All the correlations from the spin system formed by protons from C22 to C28 in the side chain are intact (Supplemental Fig. 2, COSY and TOCSY spectra). These data indicate that the second oxidation consists of an epoxide formation between C22 and C23. This assignment is further confirmed by detailed analysis of the 1H - ^{13}C HMBC spectra (Fig. 3C). The protons at C21 have all expected correlations to C22 (^{13}C at 66.2 ppm), C17 (^{13}C at 59.8 ppm), and C20 (^{13}C at 72.7 ppm). The correlation from the proton at C22 (1H at 2.86 ppm) to C20 (^{13}C at 72.7 ppm), the correlation from the proton at C23 (1H at 2.75 ppm) to C24 (^{13}C at 42.8 ppm) (Fig. 3C, inset), and the correlations from protons at C26/C27 and C28 (1H at 0.96, 0.98 ppm) to C25 (^{13}C at 33.2 ppm), C24 (^{13}C at 42.9 ppm), and C23 (^{13}C at 59.3 ppm) (Fig. 3C) are all observed as expected.

From the above data, we conclude that the basic structure of this metabolite is 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol (22*R*,23*S*-epoxyergosta-5,7-diene-3 β ,20 α -diol). We further examined our spectra and the literature to try to define the configuration at C20, C22, and C23. The many literature reports on hydroxylation at C20 by P450scc established stereospecific 20 α -hydroxylation (Mijares et al., 1967; Nes and Varkey, 1976; Corey et al., 1991). The characteristic

chemical shift for protons at C21 ($\delta = 1.29$ ppm) in this metabolite further confirms the 20 α -hydroxyl configuration (Li et al., 2010).

Considering the configuration at C22 and C23, there are two possible isomers for this metabolite: 22*S*,23*R* or 22*R*,23*S* (Fig. 4). The configurations of 22*S*,23*S* or 22*R*,23*R* would require the two protons at C22 and C23 to be in a *cis*-like (or “Z-like”) configuration and are therefore unlikely because of the original *E*-configuration of the C22=C23 double bond in the parent ergosterol. To define the configuration at C22 and C23, we measured the NOESY spectrum for this metabolite and performed molecular modeling studies using Schrödinger molecular modeling suites (Schrödinger Inc). We could not unambiguously define which isomer is the product C with these methods. Interestingly, Misharin et al. (2007) reported the total synthesis of analogs with side chains similar to product C (the only difference was the presence of the 20 α -hydroxyl group in our product C). The configuration at C22 and C23 of the epoxide ring for both isomers was unequivocally defined in their studies by total synthesis and subsequent chemical transformations. One of the key factors in defining the stereochemistry in that study was the three bond coupling constant between the protons at C23 and C24. For the similar structures corresponding to isomers A and B shown in Fig. 4, this coupling constant was 8.1 and 7.5 Hz, respectively. In product C, this coupling constant was 7.9 Hz, much closer to that of the analog corresponding to isomer A (Fig. 4). Therefore, we assigned the structure of product C as 22*R*,23*S*-epoxyergosta-5,7-diene-3 β ,20 α -diol.

Determination of the Structure of Product D as 22-Keto-23-hydroxy-22,23-dihydroergosterol. Similar to product C, product D also has a molecular weight of 428 based on the observed molecular ion of 451 $[M + Na]^+$ (Supplemental Fig. 1B), indicating that it also contains two extra oxygen atoms relative to ergosterol. Unlike in product C, all the methyl protons in product D, including protons at C21, maintain their multiplicity patterns and chemical shift ranges relative to ergosterol, indicating no hydroxylation at C20, C21, or C24-C28 (Fig. 5, A and B). The steroid core structure is also intact based on the analysis of the two-dimensional spectra (Supplemental Fig. 2; Table 1). The one-dimensional proton NMR spectrum of this metabolite indicates the loss of the double bond between C22 and C23 (Fig. 5A). 1H - ^{13}C HSQC spectrum revealed a new methine peak at 4.31 ppm for proton (^{13}C at 80.3 ppm; Fig. 5B, inset). This proton is in the same spin system with protons at C24 (1.68 ppm), C25 (1.69 ppm), and methyl protons at C26, C27, and C28 (1H at 0.99, 1.04, and 0.75, respectively), but not with protons at C20 (2.84 ppm) or C21 (1.12 ppm), based on the 1H - 1H TOCSY spectrum (Fig. 5C). Clearly, this methine proton at 4.31 (^{13}C at 80.3 ppm) is at either C23 or C22 and is attached to a carbon that also bears a hydroxy group based on its characteristic chemical shifts. From the 1H - ^{13}C HMBC spectrum, 21-CH₃ (1H at 1.12 ppm) has the expected correlations to C17 (^{13}C at 54.5 ppm), C20 (^{13}C at 45.2 ppm), and a quaternary carbon at 218.4

FIG. 5. NMR spectra of 22-keto-23-hydroxy-22,23-dihydroergosterol [3 β ,23*S*-dihydroxyergosta-5,7-dien-22-one]. A, one-dimensional proton. B, ^1H - ^{13}C HSQC. C, ^1H - ^1H TOCSY. D, ^1H - ^{13}C HMBC. E, ^1H - ^1H COSY. Full spectra are shown in supplemental figures.

ppm (Fig. 5D and its inset). This new quaternary carbon has to be C22 because of the skeleton of the side chain, and it is a characteristic carbonyl carbon. Thus, the new methine proton at 4.31 ppm must be at C23, as could be confirmed further by 1) ^1H - ^1H COSY in which the proton at C23 (^1H at 4.31 ppm) correlates to the proton at C24 (^1H at 1.68 ppm) (Fig. 5E); and 2) the presence of HMBC correlation from protons at C28 (^1H at 0.75 ppm) to C23 (^{13}C at 80.3 ppm) (Fig. 5D). The above analysis clearly indicates that the basic structure for product D is 22-keto-23-hydroxy-22,23-dihydroergosterol (3 β ,23-dihydroxyergosta-5,7-dien-22-one). We further confirmed the presence of a carbonyl group from its IR spectrum in which a relatively strong peak at a wavenumber of 1693 cm^{-1} was observed (Supplemental Fig. 3).

We also defined the configuration of product D at the C23 position by analyzing its NOESY spectrum combined with molecular modeling (Fig. 6). A close examination of the nuclear Overhauser effect (NOE) correlation from the proton at C23 (δ = 4.31 ppm) revealed two important features. First, there is no detectable NOE from this

proton to the methyl protons at C21 or C28, indicating the distance is relatively long (4 Å or longer). Second, there is a strong NOE from this proton to the methine proton at C20, indicating that they are very close in space ($\sim$ 2.5 Å). Aqueveque et al. (2005) reported the relative configuration and biological activity of a new triterpenoid, favolon B, which has exactly the same side chain as product D. Both the proton and the carbon chemical shifts in the favolon B corresponding nuclei at C23 and C22 are identical to those in product D, confirming our basic structural assignment. Based on NMR spectra analysis of favolon B, the configuration at C23 was assigned as 23*S*. This is also in agreement with the NOESY spectra of our product D. Aqueveque et al. (2005) reported the preferable conformation of favolon B in which an intramolecular hydrogen bond between the 22-carbonyl and 23-hydroxy groups in the side chain is formed. This preferred geometry results in large separations between the protons at C23 and C21 or C28 (4–5 Å, weak or no NOE) in the 23*S* isomer, which is consistent with the NOESY data for product D (Fig. 6). In contrast, a 23*R* configuration would put the proton at C23 on the same side of the

FIG. 6. Defining the stereochemistry at C23 for product D using NOESY. The proposed 23*S* configuration is consistent with experimentally observed NOE correlation, and the 23*R* configuration is inconsistent with NOE correlation.

molecule relative to the protons at C21 or C28. This would result in a much shorter distance (2.5–2.8 Å) between the protons, and therefore, a very strong NOE, rather than no NOE, would be expected (Fig. 6). These results strongly indicate a 23*S* configuration. In fact, if we consider that both product C and product D are likely produced by a common intermediate (see *Discussion*), then we would expect the configuration at C23 to remain the same (i.e., 23*S* configuration) in both metabolites. On the basis of these findings, we defined the structure of product D as 3 β ,23-dihydroxyergosta-5,7-dien-22-one.

Molecular Modeling of Ergosterol into the Crystal Structure of Human P450sc. To better understand the propensity of human P450sc to catalyze epoxidation of the ergosterol side chain, we examined the potential ergosterol structure at the active site of the crystal structure of human P450sc (Strushkevich et al., 2011). The C22=C23 double bond aligns directly below the oxygen binding site of the heme group, approximately 4.1 Å from the iron (Fig. 7). This relatively “shallow” penetration of the side chain into the active site for ergosterol compared with that of the native ligand is probably due to the constrained geometry of the double bond in ergosterol

and/or the electrostatic interaction between the electron-rich C22=C23 double bond and the heme iron. The double bond is thus the closest site for reaction with the activated oxyferryl complex, therefore, favoring epoxidation. This model also suggests that epoxidation at C22–C23 occurs before hydroxylation at C20 or C22. It is very interesting to note that on the basis of the structure of this substrate-P450sc complex, oxidation of the C22=C23 double bond will yield an intermediate epoxide metabolite having the 22*S*,23*S* configuration, which is consistent with the stereochemistry assignments in product C and D, as described earlier (note that although the additional hydroxylation at C20 does not affect the Cahn-Ingold-Prelog descriptor at C23, it will change this descriptor at C22 from 22*S* to 22*R* because the additional nearby oxygen atom alters the priority orders of groups at C22).

When we performed the docking calculation with the 22*S*,23*S*-epoxide of ergosterol in the P450sc active site, several interesting characteristics appeared. First, the overall pose of this metabolite in the active site is much closer to that of the native ligand (Fig. 7). Second, this deeper penetration of the side chain effectively puts C20

FIG. 7. Docking of ergosterol and 22,23-epoxy-22,23-dihydroergosterol into the active site of human P450sc. A, the positions of the native ligand (20*R*,22*R*-dihydroxycholesterol) and ergosterol in the active site of human P450sc in relation to the heme group. B, the position of the native ligand and 22*S*,23*S*-epoxy-22,23-dihydroergosterol (22*S*,23*S*-epoxide-ergosterol).

SCHEME 1. Rearrangement of oxiranol intermediate.

and C22 under the heme group, with similar distance to the heme (4.8 Å from the iron atom to C22 and 5.0 Å from the iron atom to C20 in this model). If this distance plays a dominant role in the rate of hydroxylation, then this model will suggest that the relative amount of products from further hydroxylation at C20 and C22 will be similar. This is indeed the case as the proportions of product C and product D are very similar (see Fig. 1). Thus, the molecular modeling, which inevitably simplifies the conceivably complicated enzymatic reaction process, does provide predictions compatible not only with the sites of oxidation, but also with the stereochemistry of the metabolites and their relative quantities.

Discussion

This study reveals that human P450_{scc} can carry out both hydroxylation and epoxidation reactions on ergosterol. Whereas epoxidation reactions by other cytochromes P450 are well-known (Meunier et al., 2004), this is the first report of an epoxidation reaction carried out by P450_{scc}. Specifically, the C22=C23 double bond of the ergosterol undergoes epoxidation. Additionally, human P450_{scc} hydroxylates the ergosterol side chain, producing 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol and 22-keto-23-hydroxy-22,23-dihydroergosterol as the major products. It is likely that the formation of the 22-keto-23-hydroxy product occurs also through an epoxide (oxirane) intermediate. After hydroxylation at C22 by P450_{scc}, the highly strained oxiranol intermediate is initially formed and rearranges to the more stable α -hydroxyketone, as illustrated in Scheme 1. Similar formation of an α -hydroxyketone in the enzyme-catalyzed hydrolysis of epoxy enol acetates via an unstable oxiranol-type intermediate has been reported (Gravil et al., 2006).

The initial formation of an oxirane intermediate before hydroxylation at C20 and C22 is also supported by the docking of both ergosterol and the oxirane intermediate into the active site of the crystal structure of human P450_{scc}. With ergosterol, the C22=C23 double bond is the closest part of the side chain to the heme iron favoring epoxide formation. After this, C20 and C22 become the closest carbons and are approximately equidistant from the iron, consistent with hydroxylation at both of these positions. Although we have not been able to identify the oxirane intermediate among the ergosterol metabolites, it could be one of the unidentified products. Specifically, it could be one of the two minor metabolites (Fig. 1, products A and B) with HPLC retention times between those of ergosterol and the two major products, 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol and 22-keto-23-hydroxy-22,23-dihydroergosterol. We collected the more abundant of these two metabolites, product B, for NMR analysis, but it proved to be a mixture of compounds that we could not separate, so structure determination was not possible. The small amount of these products suggests that intermediates in the formation of 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol and 22-keto-23-hydroxy-22,23-dihydroergosterol by human P450_{scc} remain largely enzyme-bound. This is similar to the intermediates in the metabolism of cholesterol by P450_{scc}, which remain enzyme-bound (Tuckey, 2005), but not for vitamin D₃ or D₂ metabolism where products from the first hydroxylation dissociate

and accumulate in large amounts before secondary products are observed (Guryev et al., 2003; Slominski et al., 2005a, 2006; Tuckey et al., 2008a; Nguyen et al., 2009).

Interestingly, bovine P450_{scc} metabolizes ergosterol to 17 α ,24-dihydroxyergosterol as the major product, distinct from the two epoxides seen in the present study with the human enzyme (Slominski et al., 2005b). Although it would seem that there is a notable species difference between the catalytic activities of bovine and human P450_{scc}, preliminary studies indicate that 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol and 22-keto-23-hydroxy-22,23-dihydroergosterol are among the products made by the bovine enzyme. Vitamin D₂ contains the same C22=C23 double bond in its side chain as its ergosterol precursor. It is also metabolized by bovine P450_{scc}, with the two major products being 20-hydroxyvitamin D₂ and 17,20-dihydroxyvitamin D₂, with no epoxides among the three major products identified (Slominski et al., 2006; Nguyen et al., 2009).

Our study shows that ergosterol, the vitamin D₂ precursor, is a good substrate for human P450_{scc}, with K_m and k_{cat} values almost identical to those for cholesterol. In comparing rates, it should be noted that cholesterol undergoes three oxidative reactions for its conversion to pregnenolone (Tuckey, 2005), whereas ergosterol undergoes only two oxidations for formation of its two major products, so the individual oxidations of ergosterol occur at a slightly lower rate than those of cholesterol. Because P450_{scc} is expressed in the gut for local glucocorticoid production (Fernandez-Marcos et al., 2011), P450_{scc} in this tissue is likely to be exposed to ergosterol concentrations sufficient to enable some metabolism in competition with cholesterol. In this context, ergosterol metabolites could act at the local level, as occurs for gut-produced corticosteroids (Fernandez-Marcos et al., 2011), or even enter the systemic circulation. Furthermore, when topically applied, ergosterol could serve as a substrate for cutaneous P450_{scc} (Slominski et al., 2004, 2007) with potential local antiproliferative action, as has already been demonstrated for dihydroxyergosterol in cell culture (Slominski et al., 2005b). The final fate of the two major products of ergosterol metabolism by P450_{scc}, 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol and 22-keto-23-hydroxy-22,23-dihydroergosterol, and whether these products are biologically active like some of the hydroxyergosterol products of bovine P450_{scc} (Slominski et al., 2005b), remains to be established.

Authorship Contributions

Participated in research design: Tuckey, Nguyen, Chen, Slominski, and Li.
Conducted experiments: Tuckey, Nguyen, Chen, Tieu, and Li.
Contributed new reagents or analytic tools: Tuckey, Baldisseri, and Li.
Performed data analysis: Tuckey, Nguyen, Chen, Slominski, Tieu, Zjawiony, and Li.
Wrote or contributed to the writing of the manuscript: Tuckey, Nguyen, Chen, Slominski, Zjawiony, and Li.

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