Ketoconazole and other imidazole derivatives are potent inhibitors of peroxisomal phytanic acid α-oxidation

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Abstract The imidazole antimycotics like ketoconazole, clotrimazole, bifonazole, miconazole and CO, known as powerful inhibitors of cytochrome *P*-450, are potent inhibitors of peroxisomal phytanic acid α-oxidation to pristanic acid suggesting the possible involvement of the cytochrome *P*-450 mono-oxygenase system in this oxidation. In contrast to the inhibition of the oxidation of [1-^{14}C]phytanic acid, [1-^{14}C]phytanoyl-CoA and [(2,3)-^{3}H]phytanic acid, these drugs and CO have no effect on the oxidation of [1-^{14}C]α-hydroxy phytanic acid indicating that these drugs and CO inhibit only the α-hydroxylation of phytanic acid. These studies using purified peroxisomes from liver and cultured human skin fibroblasts and Hep G2 cells clearly demonstrate that α-hydroxylation, an intermediate step in the α-oxidation of phytanic acid found to be impaired in Refsum Disease, is mediated by cytochrome *P*-450 containing enzyme.

Key words: Phytanic acid; Peroxisome (human); Antimycotics; Cytochrome *P*-450

1. Introduction

Phytanic acid (3,7,11,15-tetradecanoic acid), a saturated branched-chain fatty acid, is present in trace amounts in healthy human subjects but accumulates to excessive amounts in a number of neurological diseases [1,2]. It originates mainly from dietary sources [1] with an estimated daily dietary intake of 50–100 mg in humans. Due to the presence of the beta methyl group on its fatty acid chain it cannot be β-oxidized, but it is α-oxidized [1] to its lower homologue, pristanic acid, which is then further catabolized by β-oxidation. An abnormality in its catabolism causes accumulation of phytanic acid in tissues and body fluids of patients with Refsum disease, Rhizomelic chondrodysplasia punctata (RCDP) and in patients with defects in peroxisomal biogenesis (e.g. Zellweger syndrome, Infantile Refsum Disease and Neonatal Adrenoleukodystrophy) [1,2].

Recently, we have established that in humans α-oxidation of phytanic acid to pristanic acid takes place in peroxisomes [3–7]. Phytanoyl-CoA ligase is present in the peroxisomal limiting membrane, and the enzyme system involved in the next three steps (i.e. α-hydroxylation, dehydrogenation and decarboxylation) is present in the peroxisomal matrix [6]. Phytanoyl-CoA is synthesized on the cytoplasmic surface of the peroxisomal membrane by phytanoyl-CoA ligase, a prerequisite for phytanic acid's import into peroxisomes. Phytanoyl-CoA is then converted to phytanic acid prior to its α-oxidation to pristanic acid in the matrix of peroxisomes [7]. The α-oxidation of phytanic acid to pristanic acid involves at least three steps: α-hydroxylation to α-hydroxy phytanic acid, conversion to 2-ketophytanic acid and finally α-decarboxylation to a 19-carbon homologue, pristanic acid (Fig. 1). Based on the normal oxidation of α-hydroxy phytanic acid as compared to deficient oxidation of phytanic acid in monolayers of cultured skin fibroblasts from Refsum patients, Steinberg and associates [8] have suggested that the defect in the α-oxidation of phytanic acid in Refsum may be in the α-hydroxylation step. By using purified peroxisomes from cultured skin fibroblasts of control and patients with Refsum disease we have demonstrated that α-oxidation of phytanic acid to pristanic acid is defective in peroxisomes of Refsum disease [5]. However, at present very little is known about the biochemical characteristics of phytanic acid α-hydroxylase, which appears to be a key enzyme of phytanic acid catabolic pathway. In this manuscript, we report that imidazole antimycotics, potent inhibitors of cytochrome *P*-450, and CO inhibit the oxidation of phytanic acid but not of α-hydroxy phytanic acid demonstrates the involvement of cytochrome *P*-450 in the α-hydroxylation, the step which is defective in Refsum disease.

2. Materials and methods

Nycodenz was obtained from Accurate Chemical and Scientific Corp., Westbury, NY. ATP and CoASH were purchased from P-L Biochemicals, Milwaukee, WI. Cytochrome *c*, α-cyclodextrin and FAD were purchased from Sigma Chemical Co, MI. [1-^{14}C]Phytanic acid (55 mCi/mmol) was purchased from Amersham International, Arlinghton Heights, IL. [1-^{14}C]Phytanoyl-CoA was synthesized as described [9]. [(2,3)-^{3}H]Phytanic acid was synthesized from [(2,3)-^{3}H]dihydrophytol as described by Zenger-Hain et al. [10]. [1-^{14}C]α-hydroxy phytanic acid was synthesized from [1-^{14}C]phytanic acid by the treatment of oxalyl chloride and bromine followed by alkaline hydrolysis as mentioned by Skjeldal and Stokke [11].

2.1. Isolation of peroxisomes from human liver

Peroxisomes from human liver were isolated according to the procedures described previously [4]. Briefly, the liver homogenate was first fractionated by differential centrifugation to prepare the light mitochondrial fraction, the 'lambda' fraction [12], and the peroxisomes from the lambda fraction were prepared by isopycnic equilibrium centrifugation in continuous Nycodenz gradients. Tubes (39 ml) for a Beckman JV-20 rotor were layered with 4 ml of 55% (w/v) Nycodenz and 28 ml of a continuous gradient consisting of 0–50% (w/v) Nycodenz in homogenization medium. Gradient fractions were analyzed for the following subcellular markers: cytochrome *c* oxidase for mitochondria [13], NADH cytochrome *c* reductase for microsomes [14] and catalase for peroxisomes [15]. Protein concentration was determined by the procedure of Bradford [16]. The three fractions with highest catalase activity from the gradient were pooled, dialyzed against the homogenization buffer for 1 h to lower the concentration of Nycodenz and used for further studies.

2.2. Activation and oxidation of fatty acids

The activities for phytanoyl-CoA ligase were measured as described previously [5–7]. Briefly, the reaction mixture in 0.25 ml contained

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12 μM [1-^{14}C]phytanic acid, 50 mM KCl, 5mM $MgCl_2$, 50 μM CoASH, 10 mM ATP, and 30 mM MOPS-HCl buffer, pH 7.8. The α-oxidation of [1-^{14}C]phytanic acid and [1-^{14}C]α-hydroxy phytanic acid was measured as liberated $^{14}CO_2$ as described previously [3,4]. Briefly, the reaction volume of 0.25 ml contained 12 μM fatty acid, 30 mM KCl, 5 mM $MgCl_2$, 50 μM CoASH, 10 mM ATP, 0.25 mM NADPH, 0.17 mM FAD and 20 mM MOPS-HCl buffer, pH 7.8. The assay for oxidation of [(2,3)-^{3}H]phytanic acid was done essentially as described by Zenger-Hain et al. [10].

3. Results

3.1. Effect of imidazole derivatives on α-oxidation of phytanic acid by human liver peroxisomes

Peroxisomes were purified from human liver by differential and Nycodenz density gradient centrifugation, procedures routinely used in the laboratory [4–7]. Peroxisomes in the peroxisomal peak fraction (density of 1.27 gm/ml) had approximately 95% purity with minor contamination by mitochondria (1.0–1.5%) and microsomes (2.8–3.5%) as protein. Percent purity of peroxisomal fractions was calculated according to the method described by Fujiki et al. [17]. Fig. 2 shows the effects of different concentrations of the imidazole antimycotics on the activity of phytanic acid α-oxidation in human liver peroxisomes. These drugs were able to markedly inhibit peroxisomal α-oxidation of phytanic acid in a concentration-dependent manner, and at a concentration of 5 μM inhibited α-oxidation of phytanic acid in the range of 30 to 50%. Among the four imidazole derivatives used, ketoconazole was seen to be the most potent in inhibiting α-oxidation.

To identify the metabolic step in the α-oxidation of phytanic acid that is inhibited by imidazole antimycotics we examined the effect of these compounds on the activation of phytanic acid by phytanoyl-CoA ligase (Table 1) and oxidation of the substrates for different steps of the α-oxidation pathway (Tables 2 and 3). The activation of phytanic acid to phytanoyl-CoA by phytanoyl-CoA ligase is the first and obligatory step in its catabolism in intact peroxisomes [6], but these drugs had no effect on phytanoyl-CoA ligase activity (Table 1). Table 2 shows the effect of these antimycotics and carbon monoxide (CO) on the oxidation of [1-^{14}C]phytanic acid, [1-^{14}C]phytanoyl-CoA, [(2,3)-^{3}H]phytanic acid and [1-^{14}C]α-hydroxy-

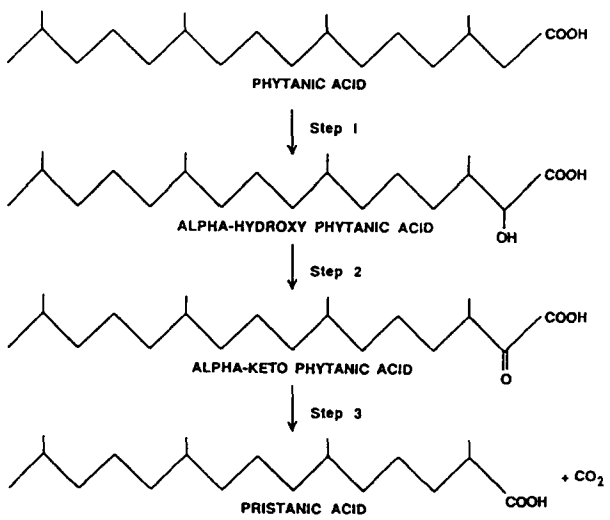

Fig. 1. Pathways of phytanic acid catabolism.

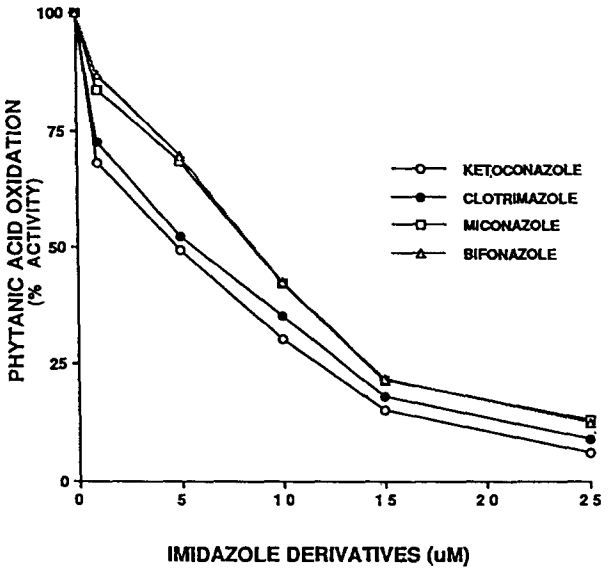

Fig. 2. Effect of different imidazole antimycotics on the α-oxidation [1-^{14}C]phytanic acid by human liver peroxisomes. Peroxisomes were isolated from human liver as mentioned in section 2. Peroxisomes were preincubated for 15 min with different amounts of imidazole derivatives. Data presented is the average of two separate determinations.

phytanic acid, which are substrates for different catabolic steps in the metabolism of phytanic acid. All these drugs at a concentration of 20 uM inhibited the oxidation of [1-^{14}C]phytanic acid and [1-^{14}C]phytanoyl-CoA by about 80 to 90%. Consistent with the fact that these imidazole antimycotics are potent inhibitors of cytochrome *P*-450 (*P*-450), these oxidations were also inhibited by CO in the range of 60 to 65%, suggesting that at least one of the steps of phytanic acid alpha-oxidation is mediated by a cytochrome *P*-450 related hemoprotein. The release of [^{3}H] from [(2,3)-^{3}H]phytanic acid as ^3H_2O [10] provides a measure of α-hydroxylation since the ^{3}H is lost from the α-position in this process (Fig. 1). The imidazole antimycotics were potent inhibitors of the release of ^{3}H from [(2,3)-^{3}H]phytanic acid (Table 2). However, in contrast to the potent inhibition of α-oxidation of [(2,3)-^{3}H]phytanic acid, these drugs and CO had no effect on the oxidation of [1-^{14}C]α-hydroxyphytanic acid indicating that they specifically inhibit the α-hydroxylation step of phytanic acid catabolic pathway indicating that this step is mediated by a cytochrome *P*-450 related hemoprotein.

Table 1
Effect of different imidazole antimycotics on activation of phytanic acid by human liver peroxisomes

Inhibitors (20 μM)	Activation (Phytanoyl-CoA ligase) (nmol/min/mg protein)
Control	7.54 ± 1.22
Ketoconazole	7.37 ± 0.95
Clotrimazole	7.70 ± 1.52
Miconazole	7.43 ± 1.46
Bifonazole	7.66 ± 0.95

Human liver peroxisomes were preincubated with different imidazole antimycotics for 15 min and phytanoyl-CoA ligase activity were measured as mentioned in the methods section. Results were expressed as mean ± S.D. of three different experiments.

Table 2
Effect of imidazole derivatives and carbon monoxide (CO) on phytanic acid α-oxidation by human liver peroxisomes

Inhibitors	Percent activity			
	[1-^{14}C]Phytanic acid	[1-^{14}C]Phytanoyl-CoA	[(2,3)^{3}H]Phytanic acid	[1-^{14}C]α-Hydroxy phytanic acid
Ketoconazole (20 μM)	10.5 ± 1.6	9.2 ± 1.9	16.6 ± 2.9	82.9 ± 10.2
Clotrimazole (20 μM)	11.3 ± 2.1	9.7 ± 1.2	15.8 ± 1.4	86.4 ± 7.8
Miconazole (20 μM)	12.8 ± 1.9	11.9 ± 2.5	17.2 ± 2.8	89.8 ± 11.3
Bifonazole (20 μM)	13.2 ± 2.8	12.4 ± 1.8	17.6 ± 3.1	90.6 ± 8.9
CO	37.3 ± 5.2	38.6 ± 4.5	41.7 ± 6.2	92.6 ± 12.5

Human liver peroxisomes were preincubated with different imidazole antimycotics for 15 min. For carbon monoxide treatment, peroxisomes were bubbled with CO for 3 min followed by O_2/CO_2 (95:5, v/v) for 30 s. Control peroxisomes were treated with N_2 in the same way with CO. Oxidations were carried out as mentioned in section 2. Results are expressed as mean ± S.D. of three different experiments. 100% activities represent 48.6 ± 6.1 pmol/h/mg for [1-^{14}C]phytanic acid, 45.9 ± 5.8 pmol/h/mg for [1-^{14}C]phytanoyl-CoA, 31.6 ± 5.3 pmol/h/mg for [(2,3)-^{3}H]phytanic acid and 34.3 ± 4.8 pmol/h/mg for [1-^{14}C]α-hydroxy phytanic acid.

3.3. *Effect of imidazole derivatives and CO on phytanic acid oxidation by human skin fibroblast and human HepG2 cells*

Since imidazole antimycotics inhibit α-oxidation of phytanic acid in peroxisomes isolated from human liver, we also examined the effect of ketoconazole on phytanic acid oxidation by cultured human skin fibroblasts and human HepG2 cells. The cultured cells were treated with ketoconazole 20 μM for 15 min and then examined for their ability to metabolize phytanic acid derivatives (Table 3). Consistent with the inhibitions found on phytanic acid oxidation in human liver isolated peroxisomes, the oxidation of [(2,3)-^{3}H]phytanic acid, [1-^{14}C]phytanic acid and [1-^{14}C]phytanoyl-CoA in both the cell lines was inhibited in the range of 75 to 85% in the presence of ketoconazole; whereas the oxidation of [1-^{14}C]α-hydroxy phytanic acid remained unaffected. These studies on human liver isolated peroxisomes and intact human cell lines (cultured skin fibroblasts and HepG2) clearly demonstrate that a *P*-450-like hemoprotein is involved in the α-hydroxylation of phytanic acid in peroxisomes.

4. Discussion

Recent studies have provided conclusive evidence for the involvement of peroxisomes in the α-oxidation of phytanic acid in humans [3–7]. Here we describe the imidazole antimycotics as inhibitors of peroxisomal phytanic acid α-oxidation, with ketoconazole being the most potent. Inhibition of the oxidation of phytanic acid or phytanoyl-CoA by imidazole antimycotics and CO as compared to the lack of such an effect on phytanoyl-CoA ligase activity and oxidation of α-hydroxyphytanic acid indicates that the phytanic acid α-hydroxylase is a cytochrome *P*-450-like hemoprotein. Characterization and complete understanding of the mechanism of α-hydroxylation of phytanic acid is important since phytanic acid accumulates in pathognomic amounts in tissues and body fluids of patients with Refsum Disease due to an abnormality in the α-hydroxylation of phytanic acid [1,2]. Activation and transport of phytanic acid are membrane phenomena; whereas other steps of α-oxidation, including the α-hydroxylation step, take place in the peroxisomal matrix [6] suggesting that the phytanic acid alpha-hydroxylase, which is a cytochrome *P*-450 like hemoprotein, is a peroxisomal matrix component. Although cytochrome *P*-450 proteins have not been purified from peroxisomes so far, a number of lines of evidence indicate the possible presence of a *P*-450 monooxygenase system in peroxisomes. The conversion of cholesterol to propionic acid [18], hydroxylation of 3-α, 7-α, 12-α-trihydroxy, 5-β cholestane to cholestanetetrol [19], and the synthesis of cholesterol from mevalonic acid [20] by purified peroxisomes supports the presence of *P*-450 monooxygenase systems in peroxisomes. Additionally, the presence of cytochrome b_5, NADH cytochrome *c* (b_5) reductase and NADPH cytochrome *P*-450 reductase in peroxisomes [21] has been described recently. Moreover, we have identified proteins that react with antibodies against *P*-450 2E1 and *P*-450 1A1 in rat liver peroxisomes [22].

Ketoconazole and other imidazole derivatives have long been used as effective therapeutic agents in the treatment of mycotic infections [23]. At a single oral dose of 200 mg/day, ketoconazole inhibits the *P*-450 enzyme-dependent biosynthesis of ergosterol from lanosterol in fungi, leading to their eventual destruction [23,24]. Because ketoconazole can be administered orally and has a broad-spectrum of activity against both superficial and deep mycoses, this imidazole derivative has become widely used as an antifungal agent [23]. In recent years it has also become apparent that ketoconazole also inhibits steroidogenesis [23]. In high doses (400 mg three times a day),

Table 3
Effect of ketoconazole on α-oxidation of phytanic acid by cultured skin fibroblasts and human Hep G2 cells

Cells	Ketocanazole (20 μM)	Rate of Oxidation (pmol/h/mg protein)			
		[1-^{14}C]Phytanic acid	[1-^{14}C]Phytanoyl-CoA	[(2,3)^{3}H] Phytanic acid	[1-^{14}C]α-hydroxy phytanic acid
Skin Fibroblasts	without	16.8 ± 3.2	18.5 ± 2.3	13.2 ± 1.9	10.9 ± 2.4
	with	2.92 ± 0.52	2.87 ± 0.41	3.05 ± 0.55	9.56 ± 1.85
HepG2	without	22.3 ± 2.5	20.9 ± 3.8	18.6 ± 2.8	14.1 ± 2.3
	with	3.1 ± 0.2	3.2 ± 0.4	4.3 ± 0.5	12.2 ± 1.8

Oxidations were carried out in cells suspended in Hank's Balanced Salt Solution (HBSS) as mentioned in the methods section. Cells were preincubated with Ketoconazole for 15 min.

it inhibits *P*-450-dependent biosynthesis of testicular and adrenal androgens, and therefore is applied for the treatment of diseases such as advanced prostatic cancer, precocious puberty, hirsuitism and Cushing's syndrome [23,25]. However, its several side effects like gastrointestinal disturbances, muscle fatigue, dry skin, mucosa, nail dystrophy and symptoms of hypervitaminosis A restrict its routine use in these diseases [23]. The inhibition of phytanic acid oxidation by these drugs has identified another possible side effect of imidazole antimycotics. Detailed information regarding the effects of these drugs on mammalian biochemical pathways are important in terms of their clinical utility. Accumulation of phytanic acid in Refsum Disease is neurological degeneration and retinal changes consistent with retinitis pigmentosa [1,2]. The morphological changes similar to the ones observed in Refsum Disease in retinal pigment epithelial cells in an in vitro assay system when these cells were incubated with phytanic acid at concentration comparable to the levels found in affected humans support a role of excessive phytanic acid in cellular toxicity [26].

In summary, we have demonstrated that imidazole antimycotics are potent inhibitors of peroxisomal phytanic acid α-oxidation, and that peroxisomal phytanic acid α-hydroxylase is a cytochrome *P*-450 related hemoprotein.

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