Selective inhibition of mitochondrial 27-hydroxylation of bile acid intermediates and 25-hydroxylation of vitamin D_3 by cyclosporin A

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It was demonstrated recently that cyclosporin A blocks bile acid synthesis in cultured rat and human hepatocytes by specific inhibition of chenodeoxycholic acid formation. The site of inhibition was found to be the 27-hydroxylation of cholesterol catalysed by the liver mitochondrial 27-hydroxylase [Princen, Meijer, Wolthers, Vonk and Kuipers (1991) Biochem J. **275**, 501–505]. In this paper the mechanism by which cyclosporin A blocks mitochondrial 27-hydroxylation was further investigated. It is shown that cyclosporin A inhibited 27-hydroxylation of bile acid intermediates, depending on their polarity. In isolated rat liver mitochondria, 27-hydroxylation of cholesterol was dose-dependently blocked by the drug, giving half-maximal inhibition at 4 μM, whereas 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol was not affected. A similar observation was made using electrophoretically homogeneous cytochrome *P*-450$_{27}$ isolated from rabbit liver mitochondria, excluding the possibility that cyclosporin A interfered with transport of substrates into the mitochondrion. Kinetic studies showed that inhibition of the 27-hydroxylation of cholesterol by cyclosporin A was of a non-competitive type. The drug also inhibited the 25-hydroxylase activity towards vitamin D_3, catalysed by the same enzyme preparation, to the same extent as 27-hydroxylation of cholesterol. These results suggest that cyclosporin A may interfere with binding of cholesterol, but not of 5β-cholestane-3α,7α,12α-triol, to the active site of the enzyme. These data provide an explanation for the selective inhibition of chenodeoxycholic acid synthesis.

INTRODUCTION

The conversion of cholesterol into bile acids is the major pathway for degradation and excretion of cholesterol in mammals [1,2]. According to current concepts, formation of bile acids starts with modifications of the sterol nucleus in cholesterol, which are thought to precede oxidation of the side chain. From both *in vivo* and *in vitro* investigations, predominantly with rats, the major pathway for synthesis of cholic and chenodeoxycholic acids was established in the 1970s and was shown to involve 7α-hydroxylation of cholesterol as a first and rate-limiting step (for reviews see [1] and [2]). However, next to this quantitatively important route for the production of bile acids, evidence has been provided for the existence of an alternative route, starting with the introduction of a hydroxy group at C-27 in the side chain of cholesterol by the mitochondrial 27-hydroxylase [3–6]. (In accordance with IUPAC sterol nomenclature and the stereochemistry of the reaction [7,8] the enzyme catalysing 27-hydroxylation of bile acid intermediates will be denoted 27-hydroxylase, although it has been referred to as 26-hydroxylase in most previous work.) This alternative pathway leads predominantly to formation of chenodeoxycholic acid in rat and man [3–6]. It has been suggested that the 27-hydroxylase pathway is particularly important in conditions in which the activity of cholesterol 7α-hydroxylase is low [5,6].

In a recent paper, Princen et al. [6] showed that bile acid biosynthesis was dose-dependently decreased by the immunosuppressive drug cyclosporin A (CsA) in monolayer cultures of rat and human hepatocytes. The inhibition was found to be the result of a strong decrease in the production of chenodeoxycholic acid. It was concluded that the site of inhibition was the liver mitochondrial 27-hydroxylase catalysing the 27-hydroxylation of cholesterol. It was also shown that the cholesterol 7α-hydroxylase activity was not affected by the drug. These findings were interpreted to indicate that the pathway starting with initial 27-hydroxylation of cholesterol contributed substantially to the formation of bile acids in hepatocytes [6].

However, the mitochondrial 27-hydroxylase is not only able to convert cholesterol into 27-hydroxycholesterol. The enzyme has a broad substrate specificity, catalysing 27-hydroxylation of various C_{27} steroids [9–11]. The purified cytochrome *P*-450$_{27}$ has been extensively characterized [12–14] and the cDNA encoding the enzyme has been isolated [15,16]. Besides 27-hydroxylation of C_{27} steroids, the purified enzyme is active in the 25-hydroxylation of vitamin D_3 [13]. This is the first step in the bioactivation of vitamin D_3 [17]. The question whether the two hydroxylations are catalysed by the same or different mitochondrial cytochromes *P*-450 has not been finally resolved. Experiments with a monoclonal antibody against cytochrome *P*-450$_{27}$ showed that the antibody could bind to and decrease the 27-hydroxylase activity but not the 25-hydroxylase activity, which indicates that different species of liver mitochondrial cytochrome *P*-450 are involved in the two hydroxylations [14].

The question was asked why CsA selectively inhibits chenodeoxycholic acid synthesis and not formation of cholic acid, since a mitochondrial 27-hydroxylation is involved also in this pathway.

To investigate further the mechanism by which CsA inhibits the mitochondrial 27-hydroxylase, we have studied the effect of CsA on 27-hydroxylation of various bile acid intermediates using isolated rat liver mitochondria and using electrophoretically homogeneous cytochrome *P*-450$_{27}$ from rabbit liver mitochondria.

Abbreviations used: CsA, cyclosporin A; DMSO, dimethyl sulphoxide.
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EXPERIMENTAL

Materials and animals

25-Hydroxy[23,24-^{3}H(n)]vitamin D_3 (107 Ci/mmol) and [4-^{14}C]cholesterol (60 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks, U.K. 5-[4-^{14}C]Cholestene-3β,7α-diol (60 Ci/mol) [18], 7α-hydroxy-4-[7β-^{3}H]cholesten-3-one (3 Ci/mol) [9,12] and 5β-[7β-^{3}H]cholestane-3α,7α-diol (500 Ci/mol) [9,12] were prepared as described previously. 5β-[7β-^{3}H]Cholestane-3α,7α,12α-triol (500 Ci/mol) was synthesized as described by Cronholm and Johansson [19]. Vitamin D_3 was obtained from Sigma Chemical Co. CsA powder was a gift from Sandoz Ltd., Uden, The Netherlands. All other chemicals were either reagent grade or have been described previously [9,12,13].

Male Wistar rats (250–350 g) were used for preparation of mitochondria, and male rabbits of the New Zealand strain weighing 2–3 kg for isolation of cytochrome *P*-450$_{27}$. Both species were maintained on standard chow and water *ad libitum*. The rat chow contains 22.4% protein, 6.5% fat and 58.6% carbohydrate, as described previously [20]. Institutional guidelines for animal care were observed in all experiments.

Preparation of mitochondria and mitochondrial 27-hydroxylase assays

Rats were killed by decapitation, and liver homogenates (20%, w/v) in 250 mM sucrose/10 mM Tris/HCl, pH 7.4, were prepared at 4 °C with a Potter–Elvehjem homogenizer equipped with a loosely fitting pestle. Mitochondria were isolated as described by Andersson et al. [21], resuspended in the same buffer at 15–25 mg/ml, frozen in small portions and stored at −80 °C.

27-Hydroxylation of bile acid intermediates in isolated mitochondria was determined as described by Björkhem et al. [11]. The assays are based on measurement of the amount of radioactive substrate converted into its 27-hydroxylated product, separated by t.l.c. CsA was added to the assay mixture dissolved in dimethyl sulphoxide (DMSO), giving a final concentration of 0.67% (v/v). This concentration of DMSO had no effect on 27-hydroxylation of the bile acid intermediates used.

Protein and cholesterol were assayed by the methods of Lowry et al. [22] and Gamble et al. [23] respectively.

Isolation of cytochrome *P*-450$_{27}$, cytochrome *P*-450$_{7\alpha}$ and hydroxylation assays

An electrophoretically homogeneous preparation of cytochrome *P*-450$_{27}$ was purified from rabbit liver mitochondria as described previously [12–14]. Cytochrome *P*-450 catalysing 7α-hydroxylation of cholesterol was partially purified from pig liver microsomes and was a generous gift from Dr. Anders Toll, Uppsala, Sweden. Assay of cytochrome *P*-450 as well as preparation and assay of ferredoxin and ferredoxin reductase have been previously described [12].

Incubations were carried out for 20 min at 37 °C. Cholesterol (25 nmol), 5β-cholestane-3α,7α,12α-triol (62.5 nmol) and vitamin D_3 (25 nmol; all substrates in 25 μl of acetone) were incubated with 0.1 nmol of cytochrome *P*-450$_{27}$, 2 nmol of ferredoxin, 0.2 nmol of ferredoxin reductase, 1 μmol of NADPH and 0–50 nmol of CsA, dissolved in 10 μl of acetone, in a total volume of 1 ml of 50 mM Tris/acetate, pH 7.4. The microsomal 7α-hydroxylase (0.05 nmol) was incubated for 20 min with 2 units of NADPH–cytochrome *P*-450 reductase, 5 μmol of dithiothreitol, 0.05% of Triton X-100, 1 μmol of NADPH, 0–10 nmol of CsA and 25 nmol of cholesterol in a total volume of 1 ml of 50 mM Tris/acetate, pH 7.4 containing 20% glycerol and 0.1 mM EDTA. Incubations with cholesterol and vitamin D_3 were terminated with 5 ml of trichloroethane/methanol (2:1, v/v), and incubations with 5β-cholestane-3α,7α,12α-triol were terminated with 3 ml of 96% (v/v) ethanol. Quantification of the incubation products was performed by h.p.l.c. and t.l.c. as described previously [12,13,24].

RESULTS AND DISCUSSION

Since the mitochondrial 27-hydroxylase has a broad substrate specificity, catalysing 27-hydroxylation of various bile acid intermediates [9–12], the effect of CsA on 27-hydroxylase activity towards these C_{27} steroids was investigated using isolated rat liver mitochondria. As demonstrated in Table 1, CsA inhibited the 27-hydroxylation of cholesterol, 5-cholestene-3β,7α-diol and 7α-hydroxy-4-cholesten-3-one in a dose-dependent way, whereas conversion of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol was blocked marginally at high concentrations of CsA. Thus, the potency of the CsA effect was dependent on the structure of the steroid.

A possible explanation for this phenomenon might be interference of CsA with transfer of steroids into the mitochondria. Gustafsson [25] has provided evidence for the presence of different transport mechanisms for cholesterol and 5β-cholestane-3α,7α,12α-triol through the mitochondrial membranes. In addition, other investigators have reported that CsA can interact with a component of the inner membrane of liver mitochondria, thereby influencing the permeability of the mitochondria [26]. To investigate this hypothesis, cholesterol and 5β-cholestane-3α,7α,12α-triol were incubated with increasing amounts of CsA together with electrophoretically homogeneous cytochrome *P*-450$_{27}$, ferredoxin, ferredoxin reductase and NADPH. Figure 1 shows that increasing amounts of CsA block the 27-hydroxylation of cholesterol up to 73%, but not the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol. It can be concluded from these results that the drug inhibits at the enzyme level rather than interfering with substrate transport into the mitochondrion.

Since cytochrome *P*-450$_{27}$, besides 27-hydroxylation of C_{27} steroids, also catalyses 25-hydroxylation of vitamin D_3 [13], it was considered of interest to study the effect of CsA on this reaction. Using the same purified cytochrome *P*-450, it was found that the drug inhibits 25-hydroxylase activity towards vitamin D_3 to about the same degree as it blocks the 27-hydroxylation of cholesterol (Table 2).

The finding that CsA inhibits 27-hydroxylation of cholesterol, but not 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol, might indicate that the 27-hydroxylation of these substrates is catalysed by different liver mitochondrial cytochromes *P*-450. There are, however, some important results indicating the opposite. First of all, the incubations have been performed with an electrophoretically homogeneous preparation of cytochrome *P*-450$_{27}$. Secondly, preliminary results from experiments with the monoclonal antibody specific for cytochrome *P*-450$_{27}$ and not affecting vitamin D_3 25-hydroxylation [14] showed that the antibody was able to decrease the 27-hydroxylase activity towards both 5β-cholestane-3α,7α,12α-triol and cholesterol. These results strongly argue in favour of a single cytochrome *P*-450 enzyme in liver mitochondria responsible for 27-hydroxylation of the two substrates.

Since the 27-hydroxylase activity towards 5β-cholestane-3α,7α,12α-triol is unaffected by CsA, the drug appears to affect the interaction between cholesterol and cytochrome *P*-450$_{27}$. A possible explanation could be that CsA binds to cholesterol and inhibits the substrate from binding to cytochrome *P*-450$_{27}$. It was

Table 1 Effect of CsA on 27-hydroxylation of bile acid intermediates in isolated mitochondria

Mitochondria were isolated as described in the Experimental section and incubated under standard conditions at 37 °C for 30 min with 3 mg of mitochondria, 125 nmol of steroid, dissolved in 25 μl of acetone, and an isocitrate-dependent NADPH-generating system, which was added after a preincubation period of 5 min, in a total volume of 1.5 ml of 100 mM Tris/HCl buffer, pH 7.4. CsA to the appropriate concentrations was added in 10 μl of DMSO. Values shown are means ± S.E.M. (or range when $n = 2$) for n experiments with duplicate incubations. *Significantly different ($P < 0.05$) from incubation without CsA.

	27-Hydroxylation (% of control)				
CsA (μM)	Cholesterol ($n = 5$)	5-Cholestene-3β,7α-diol ($n = 3$)	7α-Hydroxy-4-cholesten-3-one ($n = 2$)	5β-Cholestane-3α,7α-diol ($n = 2$)	5β-Cholestane-3α,7α,12α-triol ($n = 3$)
0	100	100	100	100	100
1	71 ± 4*	76 ± 7	90 ± 6	97 ± 3	98 ± 2
5	41 ± 6*	55 ± 6*	78 ± 7	97 ± 1	95 ± 3
10	28 ± 6*	38 ± 5*	72 ± 6	96 ± 5	93 ± 4
50	4 ± 1*	20 ± 2*	62 ± 7	79 ± 5	86 ± 5
Absolute synthesis rate (nmol/h per mg)	0.56 ± 0.16 ($n = 5$)	2.00 ± 0.14 ($n = 3$)	3.38 ± 0.37 ($n = 2$)	2.74 ± 0.27 ($n = 2$)	3.39 ± 0.65 ($n = 3$)

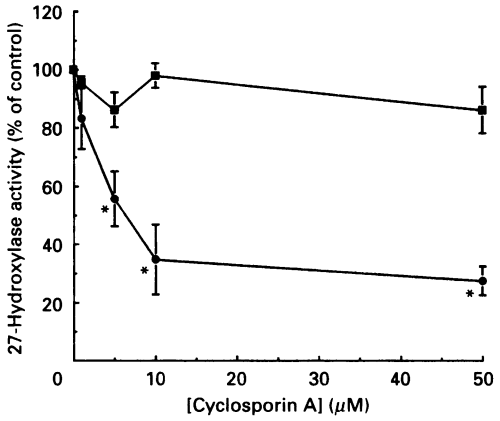

Figure 1 Effect of CsA on 27-hydroxylation of cholesterol and 5β-cholestane-3α,7α,12α-triol by cytochrome P-450$_{27}$ from rabbit liver mitochondria

Incubations were performed as described in the Experimental section. Values shown are expressed as percentages of enzyme activity in control incubations (no CsA added), and are means ± S.E.M. for three independent experiments. Absolute synthesis rates (100% values) were 3.97 ± 0.55 nmol/min per nmol of cytochrome P-450 for 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol and 0.068 ± 0.010 nmol/min per nmol of cytochrome P-450 for cholesterol. ■, 5β-Cholestane-3α,7α,12α-triol; ●, cholesterol. *Significantly different ($P < 0.05$) from incubations without CsA.

Table 2 Effect of CsA on 27-hydroxylation of cholesterol and 5β-cholestane-3α,7α,12α-triol and 25-hydroxylation of vitamin D_3 by cytochrome P-450$_{27}$ from rabbit liver mitochondria

Incubations were performed as described in the Experimental section. Values shown are means ± S.E.M. for three independent experiments. *Significantly different ($P < 0.05$) from incubation without CsA.

	27-Hydroxylation (nmol/min per nmol of cytochrome P-450)		25-Hydroxylation of vitamin D_3 (nmol/min per nmol of cytochrome P-450)
CsA (μM)	Cholesterol	5β-Cholestane-3α,7α,12α-triol	
0	0.068 ± 0.010	3.97 ± 0.55	0.050 ± 0.002
10	0.024 ± 0.003*	3.89 ± 0.16	0.026 ± 0.007*

shown, however, that CsA has no effect on 7α-hydroxylation of cholesterol in a reconstituted system containing partially purified pig liver microsomal cytochrome P-450$_{7\alpha}$, NADPH–cytochrome P-450 reductase, 10 μM CsA and cholesterol as substrate. In a typical experiment, the rate of 7α-hydroxylation of cholesterol was 4.2 nmol/min per nmol of cytochrome P-450 in the absence of CsA and 3.9 nmol/min per nmol in the presence of CsA (10 μM). This result does not support the hypothesis of a complex between CsA and substrate and agrees with the previously reported findings on the absence of an effect of CsA on cholesterol 7α-hydroxylase activity in rat liver microsomes and homogenates with concentrations up to 1 mM CsA [6]. In the same assay system with homogenate of freshly isolated rat hepatocytes, 27-hydroxylation of cholesterol was fully inhibited at 50 μM CsA [6]. It is concluded therefore that complex-formation between cholesterol and CsA cannot be the reason for the inhibition of 27-hydroxylation of cholesterol by the drug.

To further examine the mechanism of inhibition, kinetic studies were performed with the purified P-450$_{27}$. As demonstrated in Figure 2, the data on the nature of inhibition of 27-hydroxylation of cholesterol by CsA is consistent with a non-competitive rather than a competitive inhibition. This means that CsA and cholesterol appear to bind to different sites on the enzyme. Preliminary experiments with radiolabelled CsA also indicate binding of CsA to the purified enzyme. There is no information as yet on the structure of the active site of cytochrome P-450$_{27}$. It is possible that cholesterol and 5β-cholestane-3α,7α,12α-triol are differently oriented when binding to the enzyme. It may be speculated that CsA, by binding in the neighbourhood of the sterol-binding site, changes this site in such a way that binding of cholesterol and possibly also of vitamin D_3, but not of the 5β-cholestane-3α,7α,12α-triol, is blocked.

5β-Cholestane-3α,7α,12α-triol is the main substrate for degradation of the side chain in normal cholic acid synthesis [1,2,8]. The finding that 27-hydroxylation of cholesterol is inhibited by CsA and that the conversion of 5β-cholestane-3α,7α,12α-triol is not affected, provides an explanation for the previous observation that chenodeoxycholic acid synthesis is blocked by the drug and not formation of cholic acid [6]. As is shown in Table 1, 27-

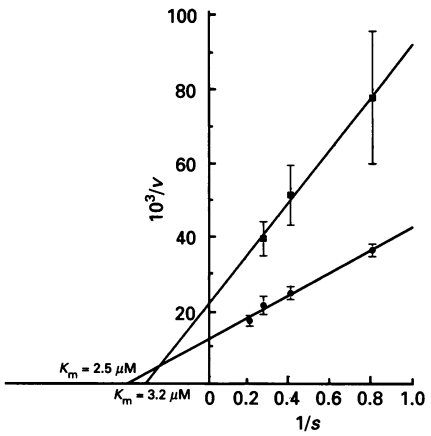

Figure 2 Lineweaver–Burk plots showing the inhibitory effect of CsA on 27-hydroxylation of cholesterol by cytochrome *P*-450$_{27}$

Incubations were performed as described in the Experimental section, with varying concentrations of cholesterol, in the presence of 10 μM (■) or no (●) CsA. *v*, velocity (pmol/min per nmol of cytochrome *P*-450); *S*, concentration of substrate (μM). Values shown are means ± S.E.M. for three independent experiments.

hydroxylation of 5β-cholestane-3α,7α-diol is also not impaired by CsA. This intermediate and 7α-hydroxy-4-cholesten-3-one are the major substrates for the mitochondrial 27-hydroxylase in the formation of chenodeoxycholic acid via the pathway starting with 7α-hydroxylation of cholesterol [1,2]. The contribution of this route to chenodeoxycholic acid synthesis in rat hepatocytes can only be estimated. The finding that formation of chenodeoxycholic acid in rat hepatocytes was blocked by 60–65% at 10 μM CsA indicates that, at least in rat hepatocytes, a substantial part of chenodeoxycholic acid synthesis is formed via the 27-oxygenation route [6]. A similar observation has been made in humans by Axelson & Sjövall [5]. These authors provide evidence that in normal subjects and particularly in patients with liver disease, in which the activity of cholesterol 7α-hydroxylase is low, the 27-oxygenation pathway is the major route to chenodeoxycholic acid. The situation may be different *in vivo* in rat. The concentration of 27-hydroxycholesterol is considerably lower in the circulation of rats than in man (I. Björkhem, unpublished work), suggesting that the pathway involving 27-hydroxycholesterol is less important in the former species. This contention is supported by the fact that cholic acid is the major bile acid in bile of control and bile-fistula rats relative to chenodeoxycholic and muricholic acids [27,28]. Nevertheless, a high dose of CsA caused a small but significant decrease in bile acid synthesis in rats with chronic bile diversion, predominantly as a result of inhibition of chenodeoxycholic acid synthesis (F. Kuipers, R. Havinga, R. J. Vonk and H. M. G. Princen, unpublished work).

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Our results do not support the previously advanced hypothesis that treatment of patients with CsA may induce formation of cholestanol and bile alcohols [29], characteristic metabolites found in patients with the inherited lipid-storage disease cerebrotendinous xanthomatosis [2,8]. It is established that the basic metabolic defect is located at the mitochondrial 27-hydroxylase [30,31]. It is clear from our experiments that 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol is not or almost not inhibited by CsA.

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