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Cyclosporin A blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid synthesis

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Bile acid synthesis, determined by conversion of [4-^{14}C]cholesterol into bile acids in rat and human hepatocytes and by measurement of mass production of bile acids in rat hepatocytes, was dose-dependently decreased by cyclosporin A, with 52% (rat) and 45% (human) inhibition at 10 μM. The decreased bile acid production in rat hepatocytes was due only to a fall in the synthesis of β-muricholic and chenodeoxycholic acids (−64% at 10 μM-cyclosporin A), with no change in the formation of cholic acid. In isolated rat liver mitochondria, 26-hydroxylation of cholesterol was potently inhibited by the drug (concn. giving half-maximal inhibition = 4 μM). These results suggest that cyclosporin A blocks the alternative pathway in bile acid synthesis, which leads preferentially to the formation of chenodeoxycholic acid.

INTRODUCTION

Cyclosporin A (CsA) is one of the most effective immunosuppressive drugs available and is widely used in organ transplantation [1,2]. The compound, a cyclic undecapeptide, selectively inhibits production of interleukin-2 by activated T-lymphocytes and prevents activation of resting T-cells by this lymphokine [1,2]. Several adverse reactions to CsA have been recognized, the most significant being nephrotoxicity. Other side-effects include hirsutism, hypertension, mild tremor and hepatic dysfunction [1,2]. The drug, which binds to [3,4] and is extensively metabolized by [5,6] the hepatic cytochrome *P*-450 system, has been shown to limit its own metabolism in rats by lowering enzyme activities associated with hepatic drug metabolism, e.g. total cytochrome *P*-450 [3,7]. CsA interferes with the hepatic cytochrome *P*-450-dependent metabolism of a variety of co-administered drugs [8]. Moreover, CsA inhibits several specific cytochrome *P*-450-dependent enzymes after a single administration *in vivo* [4] or in an assay with hepatic microsomes *in vitro* [4,9].

In view of the interaction of the drug with hepatic cytochrome *P*-450, we studied the effects of CsA on bile acid synthesis, in which several cytochrome *P*-450-dependent enzymes are involved [10]. Bile acids can be formed from cholesterol via different pathways, as has been shown in studies with radiolabelled intermediates *in vivo* and *in vitro* [10]. Although the route starting with 7α-hydroxylation of cholesterol is considered to be quantitatively the most important one for formation of bile acids, alternative pathways can exist, particularly for the synthesis of chenodeoxycholic acid. This latter bile acid is preferentially synthesized from 26-hydroxycholesterol in the rat [11–13] and man [14,15].

The present study shows that CsA inhibits bile acid synthesis in monolayer cultures of rat and human hepatocytes. The decreased synthesis in rat hepatocytes was predominantly the result of potent inhibition of the production of β-muricholic acid. Since β-muricholic acid is formed almost quantitatively from chenodeoxycholic acid in rat hepatocytes [16–18], it is suggested that CsA specifically interferes with the synthesis of chenodeoxycholic acid.

MATERIALS AND METHODS

Materials and animals

Materials used for the isolation and culture of rat and human hepatocytes, determination of bile acid synthesis from radiolabelled cholesterol, measurement of mass production of bile acids, the cholesterol 7α-hydroxylase activity and other enzyme assays were obtained from sources described previously [19–21].

CsA powder was a gift from Sandoz Ltd., Uden, The Netherlands. Hexokinase and glucose-6-phosphate dehydrogenase were from Boehringer (Mannheim, Germany). Male Wistar rats (250–350 g) were used throughout and were maintained on standard chow [21] (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. For preparation of hepatocytes and microsomes, animals were killed between 09:00 and 10:00 h. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte preparation and culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously [19–21]. Viability, as determined by Trypan Blue exclusion, was higher than 90%. The cells were seeded on 6-well cluster plates or on 60 mm-diam. plastic tissue culture dishes (Costar, Cambridge, MA, U.S.A.) at a density of 1×10^5 cells/cm^2 and were maintained in Williams E medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM-L-glutamine, 20 munits of insulin/ml, 50 nM-dexamethasone, 100 units of penicillin/ml and 100 μg of streptomycin/ml at 37 °C in an atmosphere of air/CO_2 (19:1) [19,20]. After a 4 h attachment period, and every 24 h thereafter, medium was replaced with 1 ml (wells) or 2.5 ml (dishes) of culture medium.

[4-^{14}C]Cholesterol (60 mCi/mmol), solubilized in foetal bovine serum, was added to cells after 28 h in culture up to 52 h. CsA was supplied to the medium in ethanol, giving a final concentration of 0.1% ethanol, with an equal volume of ethanol added to control cultures. This concentration of ethanol did not affect bile acid synthesis or the viability of cultured cells. After a 24 h incubation period with 0–50 μM-CsA, viability was assessed

Abbreviations used: CsA, cyclosporin A; DMSO, dimethyl sulphoxide; IC_{50}, concentration giving half-maximal inhibition; LDH, lactate dehydrogenase.

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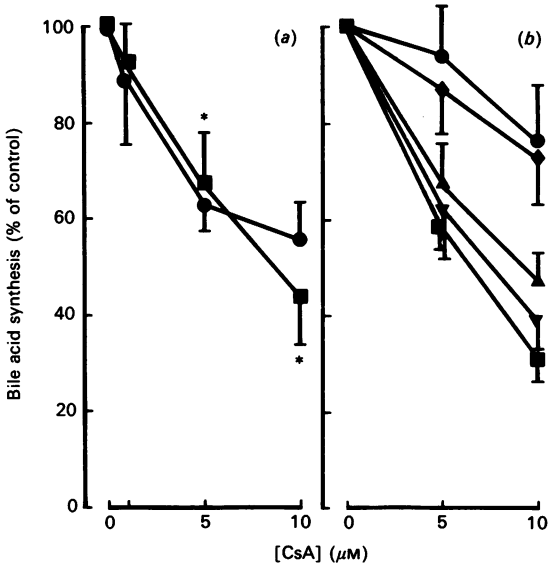

Fig. 1. Inhibition of bile acid synthesis in rat and human hepatocytes by CsA

Hepatocytes were cultured as described in the Materials and methods section. Bile acid synthesis was measured by determining conversion of 0.15 μCi of [4-^{14}C]cholesterol in 1 ml of medium/10 cm^2 of cells into bile acids in a 24 h period from 28 to 52 h (rat) or from 38 to 62 h (human) of culture. (*a*) After harvesting cells and media, and extraction according to Bligh & Dyer [40], radioactivity in the methanol/water phase was determined. The control values were 8320$\pm$2790 (mean$\pm$S.D.) and 2590$\pm$740 (mean$\pm$range) d.p.m./24 h per mg for bile acid synthesis in rat (■) and human hepatocytes (●) respectively. * indicates a significant difference ($P < 0.05$) between control and CsA-treated cells. (*b*) Effect of CsA on the synthesis of individual bile acids by rat hepatocytes. ◆, Polar bile acids (13$\pm$4%); ●, cholic acid (10$\pm$1%); ■, β-muricholic acid (54$\pm$6%); ▲, dihydroxy bile acids (14$\pm$3%); ▼, monohydroxy bile acids (9$\pm$2%). The values in parentheses are the contributions of individual bile acids to total bile acids synthesized by control hepatocytes. Differences between control and CsA-treated cells were significant ($P < 0.05$) for all individual bile acids, except for cholic acid at 5 μM-CsA. The values shown are means ($\pm$S.D.) of duplicate incubations with hepatocytes from six rats and two human liver donors.

by determination of Trypan Blue exclusion and leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium as reported before [19], and by measurement of the cellular ATP content [22].

Isolation and culture of human hepatocytes

Human hepatocytes were isolated from pieces of the left lobes of livers obtained through the Auxiliary Partial Liver Transplantation Program carried out at the University Hospital Dijkzigt in Rotterdam, The Netherlands. Consent to use the remaining non-transplanted part of the liver for scientific research was given by the Medical Ethical Committee. The livers were taken from two physically healthy organ donors (1 male and 1 female, aged 19 and 42 years), who died after severe traumatic brain injury and brain haemorrhage. Hepatocytes were isolated and cultured as described previously in detail [19,23]. Viabilities, as judged by Trypan Blue exclusion after hepatocyte isolation, were 74 and 77%. Experiments were performed with cells cultured for 38–62 h in the same medium as used for culturing of rat hepatocytes.

Determination of cholesterol synthesis and of bile acid synthesis from radiolabelled cholesterol

Cholesterol synthesis *de novo* was measured by determination of incorporation of [2-^{14}C]acetate (3 μCi, 0.1 mM) into cholesterol in cells and media during the 24 h culture period from 28 to 52 h, as described by Boogaard *et al.* [24].

Synthesis of bile acids in primary cultures of hepatocytes was determined by measuring the conversion of 0.15 μCi of [4-^{14}C]cholesterol per 10 cm^2 of cells into bile acids, accumulated during 24 h periods between 28 and 52 h (rat) or between 38 and 62 h (human) of incubation, as reported before [19,21]. After deconjugation and solvolysis [25], bile acids were separated on thin layers of silica. Areas containing bile acids were scraped off as described previously [18,21] and counted for radioactivity using a double-label program.

Quantification of mass production of bile acids in cultured rat hepatocytes

Mass production of bile acids by rat hepatocytes was measured during the 24 h culture period from 28 to 52 h as described before [21]. Shortly after addition of 2 μg of deoxycholic acid as recovery standard, bile acids in cells and media from two dishes were extracted using a Sep-Pak C_{18} cartridge, deconjugated and solvolysed [25]. Bile acids were derivatized with trifluoroacetic anhydride and hexafluoropropan-2-ol and separated and quantified on a Chrompack-Packard 438S gas chromatograph equipped with a CP Sil-19 (CB) column and flame ionization detector.

Cholesterol 7α-hydroxylase and mitochondrial 26-hydroxylase assays

Cholesterol 7α-hydroxylase activity was measured as reported in microsomes [19] or in homogenates of freshly isolated hepatocytes [20]. 26-Hydroxylation of cholesterol in isolated mitochondria was determined according to Björkhem & Gustaffson [26]. These assays are based on measurement of the amount of radioactive cholesterol converted into 7α-hydroxy[^{14}C]cholesterol or 26-hydroxy[^{14}C]cholesterol respectively. CsA was added to the assay mixture dissolved in dimethyl sulphoxide (DMSO), giving a final concentration of 1% (v/v) DMSO.

Protein and cholesterol determinations

Protein and cholesterol were assayed according to Lowry *et al.* [27] and Gamble *et al.* [28] respectively.

Statistical analysis

The statistical significance of differences was calculated using Student's *t* test for paired data with the level of significance selected to be $P < 0.05$. Values are expressed as means$\pm$S.D.

RESULTS

Effect of CsA on viability of cultured rat hepatocytes

To check whether CsA influenced the viability of hepatocytes, Trypan Blue exclusion, leakage of the cytoplasmic enzyme LDH into the culture medium and intracellular ATP content were determined in rat hepatocytes cultured from 28 to 52 h in the presence of 0–50 μM of the drug. No changes in Trypan Blue uptake (< 8%) or LDH release (< 4%) were found up to 50 μM-CsA ($n = 3$). Cellular ATP concentration was not affected at a concentration of 10 μM-CsA (control, 21.8$\pm$2.2; 10 μM-CsA, 20.5$\pm$2.0 nmol/mg of cell protein), but was significantly decreased at a concentration of 20 μM-CsA (17.7$\pm$1.1 nmol/mg of cell protein; $n = 4$). The ATP content was in good agreement

Table 1. Effect of CsA on mass production of bile acids in rat hepatocytes

Hepatocytes were cultured as described in the Materials and methods section. Bile acid synthesis was measured in the period from 28–52 h in cells and media. For control cells, values shown are means (±S.D.) of duplicate incubations of hepatocytes from six rats. For cells to which 10 μM-CsA was added, values are expressed as percentages (means ± S.D.) of bile acids synthesized in the corresponding control incubation. * indicates a significant difference ($P < 0.05$) between control and CsA-treated cells.

Bile acid	Bile acid production in control cells (μg/24 h per mg of cell protein)	Bile acid synthesis after addition of 10 μM-CsA (% of control)
Chenodeoxycholic + β-muricholic acid	0.97±0.52	36±12*
Cholic acid	0.20±0.10	100±29
Total	1.17±0.62	48±13*

with previously reported values [29]. In further experiments the CsA concentration did not exceed 10 μM.

Effect of CsA on bile acid synthesis in cultured hepatocytes

To determine the influence of CsA on bile acid synthesis, hepatocytes were incubated with [4-^{14}C]cholesterol as a substrate [17,18,21]. Addition of CsA to monolayer cultures of rat and human hepatocytes caused a dose-dependent decrease in total bile acid synthesis (Fig. 1*a*). Significant inhibition of bile acid synthesis was achieved at a concentration of 5 μM-CsA in hepatocytes from both species, whereas half-maximal inhibition was found at a concentration of approx. 10 μM-CsA (IC_{50}). In rat hepatocytes, production of β-muricholic acid and of mono- and di-hydroxylated bile acids [18] was inhibited more strongly than was the synthesis of cholic acid and of more polar compounds, which are partly formed from cholic acid [18] (Fig. 1*b*).

To see whether the drug interfered with the uptake of cholesterol by the hepatocytes, we determined the absorption of [4-^{14}C]cholesterol by the cells [19] during the 24 h incubation period with different concentrations of CsA. At 10 μM-CsA, cellular association of radiolabelled cholesterol with rat hepatocytes was increased by 46%, from 9.9±0.7% (control) to 14.5±0.9% (10 μM-CsA) of added [4-^{14}C]cholesterol/mg of cell protein (mean ± range, $n = 2$). A marked increase was also found with human hepatocytes (+21%; results not shown). It is possible, therefore, that the inhibitory effect of CsA on bile acid synthesis from exogenous labelled cholesterol might be underestimated. To exclude the possibility that the decrease in bile acid synthesis from exogenous cholesterol was caused by dilution of radiolabelled precursor cholesterol with an increased amount of newly synthesized cholesterol formed under the influence of CsA, cholesterol synthesis from [2-^{14}C]acetate was measured. Cholesterol synthesis was significantly blocked by 30±7% at 10 μM-CsA with respect to the control ($n = 3$; results not shown).

In the above-mentioned experiments, bile acid synthesis was determined by measuring conversion of pre-existing radiolabelled cholesterol into bile acids. To confirm the results obtained, we also measured mass production of bile acids by rat hepatocytes in the same period, i.e. from 28 to 52 h (Table 1). As can be seen from the S.D. values, there were large variations in bile acid synthetic rates in control hepatocytes isolated from the six individual rats. Addition of 10 μM-CsA to the cells resulted in a 52% decrease in total bile acid synthesis, which was due to a fall in the synthesis of only β-muricholic + chenodeoxycholic acid and not of cholic acid (Table 1). No increase in the proportions of formed chenodeoxycholic acid and β-muricholic acid was found with CsA (i.e. chenodeoxycholic acid comprised 5% of the sum of β-muricholic + chenodeoxycholic acid in control and CsA-treated cells), indicating that synthesis of chenodeoxycholic acid was blocked, and not conversion of chenodeoxycholic to β-muricholic acid.

Effect of CsA on microsomal 7α-hydroxylation and mitochondrial 26-hydroxylation of cholesterol

In order to elucidate the site of inhibition in the bile acid synthesis pathway, homogenates of freshly isolated rat hepatocytes [20] were incubated in the presence and absence of 50 μM-CsA. Activity of the rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase, was not affected by addition of 50 μM of the drug to the reaction mixture (control, 264±27 pmol/h per mg; 50 μM-CsA, 255±25 pmol/h per mg, $n = 2$). However, 26-hydroxylation of cholesterol, measured in this assay system, was potently inhibited at 50 μM-CsA (control, 121±27 pmol/h per mg, versus undetectable activity at 50 μM-CsA). Since the microsomal 26-hydroxylase is not active on cholesterol, and the mitochondrial 26-hydroxylase has a broad substrate specificity [10], including catalysis of the 26-hydroxylation of cholesterol [26], the effect of the drug on the 26-hydroxylation of cholesterol by isolated rat liver mitochondria was studied to confirm the experiments with homogenates of rat hepatocytes. Addition of various concentrations of CsA to the incubation mixture resulted in a strong blockade of 26-hydroxylation of cholesterol, giving 31, 44 and 62% inhibition at 1, 2.5 and 10 μM-CsA respectively, which differed significantly from the control incubation in the absence of the drug (206 pmol/h per mg). A linear relationship was observed between the logarithmic CsA concentration and inhibition of the mitochondrial 26-hydroxylation of cholesterol, giving an IC_{50} value of 4.0 μM ($n = 4$). In incubations with isolated microsomes prepared from rat liver, no inhibition of cholesterol 7α-hydroxylase activity was found with concentrations up to 1 mM-CsA ($n = 3$).

DISCUSSION

Bile acid synthesis in monolayer cultures of rat and human hepatocytes was shown to be inhibited by the immunosuppressive drug CsA. Inhibition of bile acid synthesis in cultured rat hepatocytes, as measured by different methods (mass production and conversion of pre-existing cholesterol into bile acids), was similar at 10 μM-CsA. Synthesis of cholic acid in rat hepatocyte cultures was not (mass production) or slightly (conversion of pre-existing labelled cholesterol) blocked, whereas production of β-muricholic acid and chenodeoxycholic acid were markedly decreased. The amount of chenodeoxycholic acid in the culture medium is low as a result of the very efficient conversion to β-muricholic acid by rat hepatocytes [16–18]. Furthermore, mass production of chenodeoxycholic acid was blocked to the same extent as formation of β-muricholic acid. These findings indicate that the fall in β-muricholic acid is caused by decreased chenodeoxycholic acid synthesis. In addition, since the polar bile acids are partly formed from cholic acid [18], synthesis of these compounds is also only marginally affected by CsA. Changes in the ratio of cholic acid to chenodeoxycholic acid are dependent on the activities of the 12α-hydroxylase and the 26-hydroxylases [10]. Introduction of a 26-hydroxyl group into 7α-hydroxy-4-cholesten-3-one or 5β-cholestane-3α,7α-diol, thought to be the most important substrates, prevents 12α-hydroxylation in the rat [10]. Thus inhibition of the microsomal and mitochondrial 26-

hydroxylase might favour formation of cholic acid. However, no simultaneous increase in cholic acid synthesis was found together with the decreased synthesis of chenodeoxycholic acid, making this possibility less likely.

An alternative explanation for changes in the relative proportions of cholic acid and chenodeoxycholic or β-muricholic acids can be the existence of another pathway in the formation of bile acids, in addition to the major pathway involving initial 7α-hydroxylation of cholesterol [10,30], as has been suggested by several investigators to occur in the rat, hamster, rabbit and man [11–15,31]. This route may involve initial 26-hydroxylation of cholesterol by mitochondria, leading predominantly to the formation of chenodeoxycholic acid and β-muricholic acid [11–13]. Inhibition of mitochondrial 26-hydroxylation would shut off this pathway, thereby leaving the synthesis of cholic acid unaffected. This hypothesis is supported by our finding that CsA potently inhibited mitochondrial 26-hydroxylation of cholesterol. The almost complete absence of an effect on cholic acid synthesis is in good agreement with the observation that 26-hydroxycholesterol is poorly converted into cholic acid in the rat [11,12].

The existence of alternative pathways in the formation of bile acids in cultured hepatocytes from the rat and pig has been suggested previously by us as an explanation for the discrepancy between cholesterol 7α-hydroxylase activity and mass synthesis of bile acids [21,32]. Based on the latter observations and on the magnitude of inhibition of bile acid synthesis by CsA, we suggest that formation of bile acids via this alternative pathway may contribute substantially to total bile acid synthesis, at least in cultured rat hepatocytes. An explanation for the relative importance of this 26-oxygenation route in cultured rat hepatocytes may be the partial loss of cholesterol 7α-hydroxylase activity with culture age, as reported previously [20,21]. A potential effect of CsA in lowering cholesterol 7α-hydroxylase activity during the 28–52 h culture period would further enhance the importance of the alternative pathway. Although this possibility cannot be excluded, it is not supported by our experiments, e.g. mass production of cholic acid was not affected, and conversion of labelled cholesterol into cholic acid was only slightly affected, by CsA. In any case, no direct inhibitory effect of CsA on microsomal cholesterol 7α-hydroxylase activity was found.

The quantitative importance of the different pathways *in vivo* in the rat remains to be determined. However, the large amount of cholic acid in the bile of control and bile-fistula rats relative to chenodeoxycholic and muricholic acids [33,34] suggests that this alternative route may be less important *in vivo*. Nevertheless, a high dose of CsA caused a small but significant decrease in bile acid synthesis in rats with chronic bile diversion. As in rat hepatocytes, synthesis of chenodeoxycholic acid was predominantly blocked (F. Kuipers, R. Havinga, R. J. Vonk & H. M. G. Princen, unpublished work).

Recently, Axelson & Sjövall [35] proposed a model for the biosynthesis of bile acids in man involving two major pathways, one starting with 7α-hydroxylation and the other with 26-hydroxylation of cholesterol. These authors predict that, in normal subjects and in patients with a decreased cholesterol 7α-hydroxylase activity, the 26-oxygenation pathway can be the major pathway to chenodeoxycholic acid. Since bile acid synthesis in rat and human hepatocytes was inhibited markedly by CsA at concentrations (5–10 μM) which approximate to plasma levels of the drug commonly observed in patients (0.5–5 μM; [36–38]), administration of CsA may have consequences for chenodeoxycholic acid formation in man. It is unlikely, however, that CsA will block total bile acid synthesis in humans to the same extent as in rat hepatocytes, because the contribution of cholic acid synthesis to total bile acid production in the human liver [39] is higher than in the hepatocytes. The exact mechanism by which CsA inhibits mitochondrial 26-hydroxylation requires further investigation.

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