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Effect of cyclosporin A *in vivo* on taurocholate uptake by rat hepatocytes*

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Cyclosporin A (CsA†) is a fungal metabolite that has proved to be a potent immunosuppressant, treatment with which has resulted in the significantly improved survival of kidney, liver and heart allograft patients [1, 2]. However CsA therapy is associated with various side effects, mainly affecting the kidney [3] and liver [4, 5]. The most common abnormalities related to hepatotoxicity both in humans and experimental animals are elevations of bilirubin and bile acid in blood [6, 7]. These occur without evidence of liver cell damage as necrosis and increases in serum liver enzymes are essentially absent [7–10]. The ability of CsA to cause elevation in SBA level has been linked to its ability to interfere with bile acid transport by hepatocytes [5, 11, 12] and hepatocyte membrane vesicles [13]. However, most of the data that support this mechanism are from *in vitro* studies [5, 11, 12]. Furthermore the reports that show rises in SBA in rats treated with CsA used high doses and long duration protocols [7, 14, 15]. While the nature of inhibition of CsA *in vitro* seems to be direct it is possible that there may be an indirect effect *in vivo*. To determine whether small, therapeutically relevant doses of CsA as well as large doses administered for a short duration can produce changes in the levels of serum bilirubin and SBA, it was decided to investigate the effects of different concentrations of CsA on these parameters. In addition, the uptake pattern of taurocholate by hepatocytes isolated from CsA-treated rats was also determined to see if this correlated with elevated SBA levels in similarly treated rats.

* Disclaimer: The conclusions reached and scientific views expressed in this paper are solely those of the authors. They do not necessarily reflect the views and policies of the organization in which they work.

† Abbreviations: CsA, Cyclosporin A; SBA, serum bile acids; HEPES, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase.

Materials and Methods

Chemicals. Taurocholic acid [3H(G)] (2.1 Ci/mmol) was purchased from Du Pont (Sydney, Australia). CsA was a gift from Sandoz Australia Pty Ltd (Sydney). Cremophor EL was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase was Worthington CLS type II (190 units/mg). Tris–HCl and HEPES were purchased from Calbiochem-Behring, California while Percoll was purchased from Pharmacia LKB Uppsala, Sweden. All other reagents used were of the highest purity commercially available.

Animals and experimental procedures. Male Sprague–Dawley rats weighing 300–400 g (9–15 weeks of age) from The University of Sydney Animal House were kept in a room maintained at 22°. The animals had free access to water and a standard laboratory diet. CsA dissolved in Cremophor oil vehicle was administered i.p. at doses of 0.1, 1 and 10 mg/kg/day for 4 days. Control rats were injected with an equivalent volume of Cremophor (1 mL/kg/day). Blood or liver was taken from rats under ether anaesthesia 24 hr following the last injection. Hepatocytes were isolated by the collagenase digestion technique [16, 17] with modifications including a Percoll centrifugation [18]. Viability of hepatocytes was assessed by Trypan blue exclusion immediately after preparation and was always in the range of 92 to 96%. Hepatocytes were then adjusted to a concentration of 1.4×10^6 cells/mL in a Tris–HCl buffered balanced salt solution [19]. No significant changes in hepatocyte viability were found 1 hr after the start of the uptake work.

Uptake experiments. Aliquots (1.9 mL) of the freshly isolated hepatocytes in suspension were pre-incubated at 37° with shaking at 80 oscillations/min. Uptake was initiated (at zero time) by the addition of 100 μL of radiolabelled taurocholate. The final concentrations ranged from 2 to 100 μM and the radioactivity in each flask was about 80 nCi/mL. Sampling using the 400 μL polyethylene tube/silicone oil centrifugation technique [5, 20] was carried out at 20, 40, 60, 80 and 100 seconds, and at 2, 5, 10 and 30 minutes. The polyethylene tubes were cut at the middle of the oil layer the next morning and the radioactivity in both parts was quantitated in a liquid scintillation counter (Packard Instruments Inc., Downers Grove, IL, U.S.A.). Determination of radiolabel and protein concentration [21] of the hepatocyte suspension allowed calculation of the uptake on a per milligram of protein basis.

Serum chemistry. Whole blood samples were collected from the abdominal aorta of treated rats (anaesthetized with ether) into plain tubes. Serum was stored at −20° for no longer than 5 days. Total bilirubin and bile salts were determined using spectrophotometric methods (bilirubin Unikit II was purchased from Roche Products Pty. Ltd, Australia and Enzabile from Nycomed AS, Norway). AST, ALT (SVR test kits from Behring Diagnostic Inc., NJ, U.S.A.) and AP Roche Ltd. were measured using a centrifichem (System 400 distributed by Roche).

Data analysis and calculations. Initial rates of taurocholate uptake at concentrations of 2, 5, 10, 25, 50 and 100 μM were determined from the slope in the linear range (20 to 80 sec). Kinetic parameters were determined from the initial rates of uptake of taurocholate using Michaelis–Menten kinetics. Student's *t*-test or analysis of variance and Duncan's test were used for statistical analysis. $P < 0.05$ was considered significant.

Results

In preliminary experiments (data not included) we found a clear rise in the levels of serum bilirubin and bile salts following 4–7 days of i.p. injection of rats with CsA. CsA

Table 1. Changes in serum chemistry in response to different doses of CsA

Test	Cremophor (control)	CsA (mg/kg/day) in Cremophor		
		0.1	1	10
Total bilirubin (μmol/L)	1.6 ± 0.1	1.7 ± 0.2	2.1 ± 0.1	4.6 ± 0.2*
Bile salts (μmol/L)	15.6 ± 2.4	14.4 ± 3.0	47.8 ± 7.5*	90.8 ± 6.3*
AST (I.U./L)	93 ± 8	84 ± 3	81 ± 2	79 ± 5
ALT (I.U./L)	80 ± 5	80 ± 4	80 ± 4	65 ± 6
AP (I.U./L)	363 ± 21	296 ± 54	303 ± 33	269 ± 17

The values are expressed as means ± SEM, N = 8.
* Statistically different ($P < 0.05$) from respective control.

Table 2. Effect of treatment with CsA *in vivo* on kinetic parameters* of taurocholate uptake by subsequently isolated hepatocytes

Treatment	V_{max} (nmol/min/mg protein)	K_m† (μM)
Cremophor (N = 3)	1.83 ± 0.03	10.56 ± 0.41‡
CsA + Cremophor		
0.1 mg/kg/day (N = 3)	1.97 ± 0.09	12.46 ± 0.97‡
1 mg/kg/day (N = 4)	1.82 ± 0.03	11.58 ± 0.31‡
10 mg/kg/day (N = 6)	1.76 ± 0.03	27.36 ± 0.72

* Values for K_m and V_{max} were estimated from the Eadie–Hofstee plot. Each value represents mean ± SE.
† Data were analysed using analysis of variance and Duncan's test.
‡ Values not significantly different from each other ($P < 0.05$).

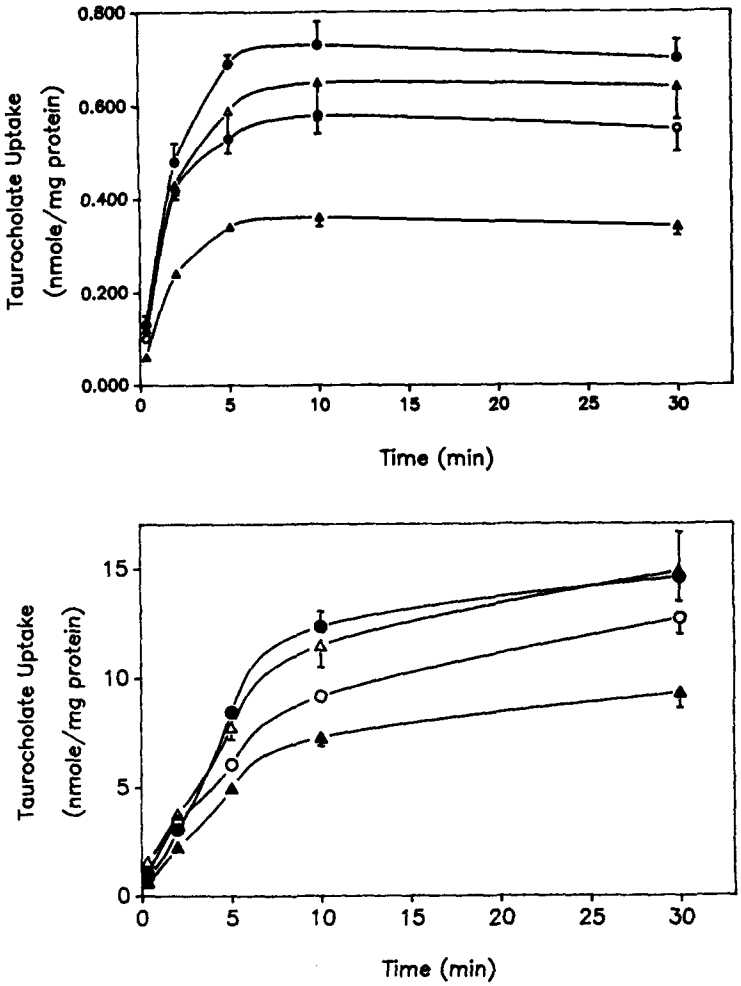

Fig. 1. Accumulation of taurocholate by hepatocytes isolated from rats treated with Cremophor (●); 0.1 mg/kg/day CsA + Cremophor (△); 1 mg/kg/day CsA + Cremophor (○); 10 mg/kg/day CsA + Cremophor (▲). Values represent the mean ± SE (N = 4). Top panel—taurocholate concentration 2 μM. Bottom panel—taurocholate concentration 100 μM. All points for 1 and 10 mg/kg/day doses of CsA are significantly different to control at the respective time ($P < 0.05$).

at 1 mg/kg/day for 2 days did not show significant changes in serum bilirubin and SBA (1.8 ± 0.3 and 19.7 ± 2.4 compared to 1.1 ± 0.2 and 17.6 ± 2.9 for the control group, respectively). The use of dimethyl sulphoxide as a vehicle instead of Cremophor EL gave similar results. We, therefore, chose 4 days (as a more convenient schedule) and Cremophor EL as the solvent (because this is used in the pharmaceutical preparation). There were no statistically significant alterations in the serum levels of AST, ALT and AP of CsA-treated rats compared to vehicle-treated rats. There was, however, a significant rise in total serum bilirubin with 10 mg/kg/day CsA and bile acid levels with 1 and 10 mg/kg/day CsA, as compared to the vehicle-treated group (Table 1).

Uptake of taurocholate. Uptake of taurocholate was linear with time (20–80 sec) at all the concentrations studied. In CsA-treated rats uptake velocity was significantly lower for all concentrations of taurocholate at 10 mg/kg/day. The relationship between uptake velocity and substrate concentration was analysed using Michaelis–Menten kinetics. Table 2 shows V_{max} and K_m for Cremophor-treated rats averaging 1.83 ± 0.03 nmol/min/mg protein and 10.56 ± 0.41 μM, respectively. After CsA treatment at 10 mg/kg, K_m was significantly different from the control group (27.36 ± 0.72 μM) while V_{max} was not. Changes in the kinetic constants for taurocholate uptake were not observed at the lower doses of CsA, however. When accumulation of taurocholate over longer incubation periods was assessed a reduction was observed at the two higher doses of CsA (Fig. 1).

Discussion

The present study demonstrates that SBA level was significantly raised with 1 and 10 mg/kg/day CsA while serum bilirubin level was only significantly raised with the higher dose. No changes were found in the serum transaminases or alkaline phosphatase even with the high dose. This is consistent with previous reports of hepatobiliary dysfunction in experimental animals [22] and humans [23] where hyperbilirubinemia and elevation of SBA are dose-dependent and occur without other significant liver pathology. A significant reduction in taurocholate

uptake by hepatocytes isolated from rats treated for 4 days with CsA was found. The pattern of inhibition was competitive in nature and has been demonstrated only with the highest dose. However, both the higher doses resulted in reduced accumulation of taurocholate by hepatocytes over extended periods of incubation. The lowest dose of CsA (0.1 mg/kg/day) did not significantly affect the uptake pattern of taurocholate compared to Cremophor-treated rats and in these rats both the level of SBA and of the serum bilirubin did not undergo any significant changes. Thus the pattern of change in SBA for treated rats is consistent with the inhibition of accumulation of bile acid by hepatocytes isolated from similarly treated animals. It is of interest to note that the pattern of inhibition *in vivo* is the same as that *in vitro*—that is, it is competitive in nature. It might be expected that hepatocytes isolated 24 hr after treatment and via a procedure including extended perfusion of the liver and then several washing steps would not contain significant amounts of CsA. However assay for CsA has shown that these hepatocytes still contain significant amounts of the drug (159 ± 120 ng/mL CsA parent compound and 205 ± 145 ng/mL CsA plus its metabolites). This is in agreement with recent reports that CsA can be detected in most tissues for at least 20 days after multiple dosing [24]. In addition, CsA is found in association with proteins of M_r 17,000 and 22,000 in the cytosol [25], where the former may be identical to cyclophilin. The possibility that CsA metabolites and not the parent molecule caused this inhibition is unlikely as the inhibitory effect of the parent compound of CsA has been demonstrated in several *in vitro* studies (e.g. Refs 5 and 12). Furthermore, it has been demonstrated recently that CsA metabolites OL17 and OL21 lack any inhibitory effect on taurocholate transport in membrane vesicles [13]. However, it seems that interference with bile acid transport is a general feature of the cyclosporin molecule regardless of differences in the structural analogs [26]. This means that both CsA parent compound and some of its metabolites play a role in the inhibition of bile acid uptake by hepatocytes.

The observation that serum bilirubin is also increased suggests that the effects of CsA may extend to the transport of this molecule as well. However, the transport system for bilirubin is distinct from that of taurocholate [27, 28] which suggests a wider ranging effect than a simple interaction with the bile salt receptor. Furthermore, it has been demonstrated recently in our laboratory that there is some specificity in the membrane effects of CsA and that it is not simply a general perturbation of membrane function [29]. The reason for hyperbilirubinemia with CsA treatment remains to be established.

In conclusion, the data show that the levels of SBA are elevated in rats treated for relatively short periods with therapeutically relevant doses of CsA. The accumulation of taurocholate by hepatocytes isolated from the treated rats is inhibited in a manner consistent with the rise in levels of SBA. This supports the contention that an interference with bile acid transport by CsA can explain the apparent cholestasis reported on administration of this drug.

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In vitro effect of clofibric acid derivatives on rat hepatic microsomal electron transport chains

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Clofibric acid (CFB*), bezafibrate (BZB) and gemfibrozil (GFB) are structurally related drugs widely used in the treatment of hyperlipoproteinemias [1–3]. Their administration to both experimental animals and humans induces a multiplicity of physiological changes: hepatomegaly, smooth endoplasmic reticulum and cytochrome P450 induction, peroxisomal proliferation [4, 5], inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase and very low density lipoprotein synthesis [6]. Nevertheless, there is no agreement about the relative contribution of the mechanisms responsible for the hypolipidemic effect.

Rat liver microsomes possess two electron transport chains [7] (Scheme 1): one of them, the microsomal NADH–cytochrome *c* reductase system, consists of cytochrome b_5 and its flavoprotein, NADH–cytochrome b_5 reductase (EC 1.6.2.2). The second system utilizes NADPH, and the electrons flow from the NADPH–cytochrome *c* reductase (EC 1.6.2.4) to cytochrome P450, and also to cytochrome b_5. Reducing equivalents flowing through cytochrome b_5 are involved in important lipid biotransformation reactions such as fatty acid chain elongation and desaturation [8]. Also, isoenzymes of cytochrome P450 are involved in ω-hydroxylation of long chain fatty acids [5].

The quality of fatty acids (chain length and number of unsaturations), either in the free form or incorporated into glycerolipids, plays an important role controlling lipoprotein metabolism [9, 10]. Given that the hypolipidemic effect of clofibric acid derivatives is not well understood, we have been interested in testing the effect of these drugs on enzymes related to the hepatic fatty acid synthesis, either *in vitro* or *in vivo*. As part of this project, we have studied the *in vitro* effect of CFB, BZB and GFB on the two microsomal electron transport chains.

Material and Methods

NADH, NADPH, cytochrome *c* (Type III from horse heart), CFB and Trizma base (Tris[hydroxymethyl] aminomethane) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.); BZB and GFB were a generous gift from Lab. Andreu (Barcelona, Spain) and Lab. Parke-Davis S.A. (Barcelona, Spain), respectively. PMSF was from Boehringer Mannheim (Mannheim, Germany), EDTA from Merck (Darmstadt, Germany), and dithiothreitol from Fluka (Buchs, Switzerland). Other general chemicals were obtained from commercial sources and were of the highest purity available.

Microsomes were prepared from the livers of Sprague–Dawley male rats (200–220 g) aged 7–8 weeks. Following 18 hr of starvation, the animals were killed by decapitation between 8 and 9 a.m. Livers were perfused with ice-cold 0.9% NaCl. The tissue was homogenized in 0.2 mM EDTA, 0.1 mM PMSF, 50 mM NaF, 0.25 M sucrose, 50 mM Tris–HCl buffer, pH 7.4, and microsomes were obtained as described previously [11], and resuspended in 0.1 M phosphate buffer pH 7.4. Protein concentration was determined by the method of Bradford [12], using bovine serum albumin as standard. NADH–cytochrome *c* reductase activity was determined by the method of Yasukochi and Masters [13]. The assay mixture contained, in final concentrations, 0.3 mM KCN, 50 μM cytochrome *c*, 100 μM EDTA, 0.1 M phosphate buffer pH 7.4, and microsomal

* Abbreviations: CFB, clofibric acid; BZB, bezafibrate; GFB, gemfibrozil; PMSF, phenylmethylsuphonylfluoride.