Cyclosporin A reduces canalicular membrane fluidity and regulates transporter function in rats

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Changes of the biliary canalicular membrane lipid content can affect membrane fluidity and biliary lipid secretion in rats. The immunosuppressant cyclosporin A is known to cause intrahepatic cholestasis. This study investigated whether cyclosporin A influenced canalicular membrane fluidity by altering membrane phospholipids or transporter expression. In male Sprague–Dawley rats, a bile-duct cannula was inserted to collect bile, and sodium taurocholate was infused (100 nmol/min per 100 g) for 60 min. During steady-state taurocholate infusion, cyclosporin A (20 mg/kg) or vehicle was injected intravenously and then bile was collected for 80 min. After killing the rats, canalicular membrane vesicles were prepared. Expression of canalicular membrane transporters was assessed by Western blotting and canalicular membrane vesicle fluidity was esti-

mated by fluorescence polarization. Cyclosporin A reduced biliary lipid secretion along with a disproportionate reduction of lipids relative to bile acids. Cyclosporin A significantly decreased canalicular membrane fluidity along with an increase of the cholesterol/phospholipid molar ratio. Only expression of the transporter P-glycoprotein was increased by cyclosporin A. Because canalicular membrane transporter expression was largely unchanged by cyclosporin A despite a marked decrease of biliary lipid secretion, transporter activity may partly depend upon canalicular membrane fluidity.

Key words: bile-salt export pump, biliary lipid, cholestasis, multidrug resistance 2, P-glycoprotein.

INTRODUCTION

Previous studies performed by our laboratory [1,2] and others [3–5] have shown that physical alterations of plasma membrane lipids (i.e. changes in fluidity and lateral packing) can influence the function of membrane carriers that mediate the transport of various nutrients, electrolytes and drugs, as well as altering membrane-bound enzyme activity. In a study using canalicular membrane vesicles (CMEVs), the carrier-mediated transport of taurocholate was shown to be modulated by alterations of membrane-lipid fluidity [6].

Cyclosporin A is a neutral highly lipophilic cyclic endopeptide and a potent immunosuppressant that has contributed significantly to progress in organ transplantation. It is a metabolite found in cultures of the fungus *Tolypocladium inflatum* Gams [7,8]. Cyclosporin A has various toxic effects, mainly on the kidneys and liver. Cholestasis is a well-known problem after liver transplantation and some investigators have reported that cyclosporin A inhibits bile-acid secretion [9–11]. Other investigators have reported that cyclosporin A inhibits the secretion of biliary components such as phospholipids, cholesterol and bilirubin [12–14]. Therefore, it has been suggested that cyclosporin A not only causes a decline in bile-acid-dependent bile flow, but also decreases bile-acid-independent bile flow.

Previous investigations of the inhibitory effect of cyclosporin A on biliary secretion have suggested that it may involve direct *cis*-inhibition of the canalicular membrane transporters [i.e. P-glycoprotein (Pgp), multidrug resistance 2 (Mdr2), multidrug resistance-associated protein 2 (Mrp2) and bile-salt export pump (Bsep)], or a decrease of membrane fluidity [15–18]. Preliminary studies have shown that basolateral liver plasma-membrane vesicles from cyclosporin A-treated rats exhibit (i) decreased membrane fluidity, (ii) an increased cholesterol/phospholipid

molar ratio and (iii) a decreased V_{max} for taurocholate transport [19]. Rossaro et al. [20,21] used ¹⁹F-NMR to demonstrate that cyclosporin A affects the inner leaflet of the membrane bilayer, altering both its packing and its motion, and can thus change membrane fluidity and various protein–lipid interactions.

The present study investigated whether cyclosporin A alters the fluidity and lipid composition of the biliary canalicular membrane, as well as whether it influences canalicular membrane-transporter function.

MATERIALS AND METHODS

Chemicals

Cyclosporin A (Sandimmune®) and Cremophor EL® were provided by Novartis Pharmaceutical Co. (Basel, Switzerland). Sodium taurocholate was purchased from Sigma (St. Louis, MO, U.S.A.), and 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Polyclonal rabbit antibodies against Mrp2 (K3, dilution 1:1000) and polyclonal antibodies against Bsep (K12, dilution 1:1000) were kindly provided by Dr M. Muller (Groningen Institute for Drug Studies, University of Groningen, Groningen, The Netherlands). Mouse monoclonal antibody C219 (dilution 1:1000; Signet Laboratories, Dedham, MA, U.S.A.) was used to detect all Pgps. Mouse monoclonal antibody 6/1G (dilution 1:300; Chemicon International, Temecula, CA, U.S.A.) was used to detect Mdr2.

Animal model and experimental protocol

Adult male Sprague–Dawley rats (300–350 g; Hiroshima Jikken Doubutsu, Hiroshima, Japan) were housed in common cages and given free access to food and water before the study. The rats

Abbreviations used: CMEV, canalicular membrane vesicle; DPH, 1,6-diphenyl-1,3,5-hexatriene; Pgp, P-glycoprotein; Mdr2, multidrug resistance 2; Mrp2, multidrug resistance-associated protein 2; Bsep, bile-salt export pump.

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were anaesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). After opening the abdomen, the bile duct was cannulated with polyethylene tubing to allow sampling of the bile. The left femoral vein was catheterized for administration of solutions. The catheters used (PE10) were purchased from Nippon Becton Dickinson Co. (Tokyo, Japan). Following surgery, the rats were placed in restraining cages, and sodium taurocholate dissolved in 0.9% NaCl was infused at the rate of 100 nmol/min per 100 g of body weight with a flow rate of 0.4 ml/h per 100 g of body weight until the end of the experiment. Bile samples were collected over 20 min periods. After an equilibration period of 25–30 min, bile was collected into pre-weighed tubes for 60 min (three 20 min collections). The last three samples of this basal period were kept for analysis and the mean values calculated were defined as baseline values. The animals then received 1 ml (over 50–60 s) of isotonic saline, cyclosporin A vehicle (Cremophor EL®/absolute ethanol/saline at 1:1:18, by vol.) or 20 mg/kg of cyclosporin A. Subsequently, the rats were killed by an intravenous injection of sodium pentobarbital and their livers were excised, weighed and prepared for the studies on liver membranes.

The study protocol was approved by the Hiroshima Animal Care and Use Committee, and the animals received humane care in compliance with the directives of the National Research Council as outlined in [21a].

Isolation of liver membranes

The excised liver was placed in ice-cold 0.25 M sucrose (9:1, v/w) containing 10 mM Hepes/Tris buffer (pH 7.4) and 0.2 mM CaCl_2 (medium A), and was homogenized at 4 °C in a Dounce homogenizer. CMEVs were prepared by nitrogen cavitation and calcium precipitation, as described by Inoue et al. [22]. In brief, 100 mM EDTA was added to the liver homogenate to a final concentration of 1 mM. After centrifugation for 10 min at 1800 *g*, the pellet and fluffy layer were collected, resuspended in medium A, and centrifuged at 3000 *g* for 10 min. Then the pellet was suspended in ice-cold medium A, and was placed in a high-pressure chamber (Parr 4645, Parr Instrument Co., Moline, IL, U.S.A.) that was equilibrated with nitrogen at 116 kPa for 15 min before the pressure was suddenly released. The resulting homogenate was added to ice-cold medium A containing 14 mM CaCl_2 and the mixture was stirred for 10 min at 0 °C. After centrifugation for 20 min at 7600 *g*, the supernatant was collected and centrifuged for 20 min at 4700 *g*. Then the pellet was resuspended in ice-cold medium A and centrifuged again for 30 min at 47000 *g*. The resulting pellet was resuspended in medium A, flushed with nitrogen, sealed and stored at –70 °C until further analysis.

The activities of two CMEV marker enzymes, leucine aminopeptidase and γ -glutamyltranspeptidase, were measured with commercially available kits (LAP C-test Wako and γ -GTP C-test Wako; Wako Junyaku Kogyo, Osaka, Japan). Compared with the crude liver homogenates, the activities of the respective enzymes showed 25-fold and 30-fold increases in CMEVs.

Analytical procedures

Total biliary bile acids were measured enzymically using 3- α -hydroxysteroid dehydrogenase [23]. The cholesterol concentration in the bile was measured using a commercial kit after extraction of lipids with diethyl ether [24]. The phospholipid concentration in bile was measured directly, as described by Bartlett [25].

Membrane lipids were extracted by the method of Bligh and Dyer [26]. The chloroform layers were pooled, washed with an

equal volume of methanol/water (1:1, v/v), and evaporated to dryness. Then the residue was dissolved in 100 μl of 10% Triton X, and aliquots (20 μl) were taken for determination of the phospholipid and total cholesterol concentrations using a commercial enzymic method. Total protein in the liver homogenates and membrane subfractions was measured with a commercially available kit (Coomassie Brilliant Blue dye-binding assay, Bio-Rad Laboratories, Richmond, CA, U.S.A.) [27].

Membrane fluidity was estimated by the DPH fluorescence depolarization method. In brief, 2×10^{-3} M DPH in tetrahydrofuran was diluted 1000-fold with vigorous stirring for 10 min at 25 °C and a stable aqueous suspension of 2×10^{-6} M DPH was obtained. Then a CMEV suspension (100 μg of protein/ml) was mixed with an equal volume of the DPH suspension and incubated at 37 °C in the dark for 45 min (the time required for the maximal fluorescence intensity to be reached) with gentle shaking. Steady-state fluorescence polarization measurements were performed using a Hitachi F2000 spectrofluorometer (Hitachi, Tokyo, Japan) equipped with a circulating-water bath that was set at 37 °C. The excitation and emission wavelengths were 360 and 430 nm, respectively. The emission intensity of vertically polarized light was detected by analysers oriented parallel (I_{vv}) or perpendicular (I_{vh}) to the plane of excitation, and fluorescence polarization (P) was calculated by the following equation: $P = (I_{vv} - I_{vh}) / (I_{vv} + I_{vh})$. The polarization values were corrected for light scatter according to the method of Lentz et al. [28]. The polarization value of DPH fluorescence was used as a measure of membrane fluidity, since increased anisotropy indicates a decrease of phospholipid acyl chain motion within the membrane [29]. All determinations were performed in triplicate.

Western-blot analysis

Canalicular membrane proteins (50 μg) were separated by SDS/PAGE [30], and were transferred to nitrocellulose membranes (Millipore, Bedford, MA, U.S.A.) using a tank-blotting system according to the manufacturer's instructions (Bio-Rad Laboratories). After transfer to the membranes, blots were blocked for 2 h at room temperature with 5% low-fat powdered milk dissolved in Tris-buffered saline containing 0.05% Tween 20, and then were incubated overnight at 4 °C with the first antibody. Immune complexes were detected using alkaline phosphatase-conjugated anti-rabbit IgG or rabbit anti-mouse IgG (dilution 1:3000) according to the manufacturer's instructions (Bio-Rad Laboratories). Detection of membrane transporters was performed by comparison with the following standards: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa) and ovalbumin (43 kDa). Then the immunoreactive bands on the membranes were quantified by densitometric analysis.

Statistical analysis

Data are expressed as means $\pm$ S.D. Statistical analysis was performed using the Mann–Whitney U test and $P < 0.05$ was defined as indicating significance.

RESULTS

Effect of cyclosporin A on biliary secretion

Injection of the vehicle did not have an observable effect on biliary secretion when compared with the injection of isotonic saline (results not shown). However, administration of cyclosporin A reduced bile flow and bile-acid secretion by 18.4 and 19.6%, respectively. The maximum decrease was seen at 20 min

Figure 1 Effect of cyclosporin A on bile secretion in rats

Changes in bile flow and changes in bile acid, phospholipid and cholesterol secretion after intravenous (i.v.) administration (arrow) of vehicle (1 ml of saline plus Cremophor EL® and ethanol, 18:1:1, by vol., ◇) or cyclosporin A (20 mg/kg, ■). The changes relative to the baseline period (0–60 min) are shown as the means $\pm$ S.D. for four or five rats in each group. * $P < 0.05$ versus vehicle-treated rats. CsA, cyclosporin A; BA, bile acid; PL, phospholipid; CH, cholesterol.

Figure 2 Effect of cyclosporin A on bile flow and the molar ratios of bile constituents

Changes were measured after intravenous (i.v.) administration (arrow) of vehicle (◇) or cyclosporin A (20 mg/kg, ■). The percentage change relative to the mean of the baseline period (0–60 min) is shown as the mean $\pm$ S.D. for four or five rats in each group. * $P < 0.05$ versus vehicle-treated rats. BF, bile flow; BA, bile acid; PL, phospholipid; CH, cholesterol.

Figure 3 Effect of cyclosporin A on canalicular membrane lipids

Rats were given an intravenous injection of 1 ml of saline, vehicle or cyclosporin A (20 mg/kg). Values are the means $\pm$ S.D. of the cholesterol/phospholipid molar ratio (CH/PL) of canalicular membranes from four rats. * $P < 0.05$ versus controls; ** $P < 0.05$ versus vehicle-treated rats.

Figure 4 Effect of cyclosporin A on canalicular membrane fluidity

Rats were given an intravenous injection of 1 ml of saline, vehicle or cyclosporin A (20 mg/kg). Values are the mean $\pm$ S.D. of the DPH depolarization value from four rats. * $P < 0.05$ versus controls; ** $P < 0.05$ versus vehicle-treated rats.

after injection, with a return to baseline values at 80 min. Cyclosporin A also reduced phospholipid and cholesterol secretion by 50.6 and 50.2 %, respectively. Maximum decreases in secretion were observed at 40 min after injection (20 min later than for bile flow and bile-acid secretion), and the recovery to baseline levels was also delayed (Figure 1).

Effect of cyclosporin A on bile flow and on the molar ratios of bile constituents

Figure 2 shows the changes of the bile flow/bile acid ratio and the phospholipid/bile acid, cholesterol/bile acid and cholesterol/phospholipid molar ratios after administration of cyclosporin A or its vehicle. The bile flow/bile acid ratio and the cholesterol/phospholipid ratio did not show significant differences between vehicle and cyclosporin A treatment. However, the ratios of cholesterol and phospholipids to bile acids were significantly lower after cyclosporin A treatment than after vehicle treatment.

Figure 5 Effect of cyclosporin A on canalicular membrane transporters

Rats were given an intravenous injection of 1 ml of saline, vehicle or cyclosporin A (20 mg/kg). Control-band intensity was set to 1; means $\pm$ S.D. are shown. * $P < 0.05$ versus control; ** $P < 0.05$ versus vehicle-treated rats.

Effect of cyclosporin A on canalicular membrane lipids and membrane fluidity

Analysis of canalicular membrane lipids showed an increase of the cholesterol/phospholipid molar ratio by 21.6 % after cyclosporin A treatment compared with vehicle-treated and saline-treated rats (Figure 3). Canalicular membrane fluidity was assessed by the DPH fluorescence depolarization method. After cyclosporin A treatment, the polarization value was significantly higher (+11.0 %) than in vehicle-treated or saline-treated rats, indicating that cyclosporin A decreases canalicular membrane fluidity (Figure 4).

Western-blot analysis of canalicular membrane transporters

The changes of membrane transporters in CM_{Ev} were quantified by densitometry and expressed as a percentage of the level in the corresponding untreated rats. Expression of Mdr2, Mrp2 and Bsep was not altered by cyclosporin A, whereas expression of Pgps was increased in cyclosporin A-treated rats by 70 % compared with vehicle-treated rats and untreated rats (Figure 5).

DISCUSSION

The present study clearly demonstrated that cholestasis was induced by cyclosporin A. The decrease in bile flow mainly resulted from a specific reduction of bile-acid-dependent bile flow, since the time-course profile of bile-acid output was very similar to that of bile flow. Thus cyclosporin A had a negligible influence on bile-acid-independent bile flow. No cholestasis was noted in vehicle-treated rats, although previous investigators have reported a cholestatic effect of Cremophor EL® [31]. Cyclosporin A is metabolized extensively by cytochrome P450 enzymes, with subsequent biliary (90 %) and urinary (6 %) excretion [7,8]. Cytochrome P450 inducers, such as phenobarbital, have been previously shown to abolish the decrease in bile flow observed after cyclosporin A administration [32], suggesting that metabolites of this drug have no effect on bile secretion.

Accordingly, we could ignore the influence of metabolites on the present findings.

This study also showed that cyclosporin A induced overexpression of Pgps, in agreement with Jette et al. [33,34]. In contrast, expression of Mdr2, Mrp2 and Bsep, which are important carrier proteins for biliary secretion, was not induced. Saeki et al. [35] reported that human Pgp was involved in the transport of cyclosporin A *in vitro*, and thus overload of this drug might enhance Pgp expression. However, the intracellular events leading to overexpression of Pgp still remain to be established.

We also observed that cyclosporin A increased the cholesterol/phospholipid ratio and reduced the membrane fluidity of CMEVs. Such changes may support the previous report by Whittington et al. [19] that the inhibition of biliary secretion by cyclosporin A is due partly to its membrane-rigidifying effect. Whittington et al. [19] showed that cyclosporin A increased the cholesterol/phospholipid ratio and reduced the membrane fluidity of hepatocyte basolateral membranes, whereas Azer and Stacey [36,37] reported that the drug interfered with the uptake of bile acids by human hepatocytes. It has also been suggested that passive influx of cyclosporin A could induce basolateral membrane damage in hepatocytes as a result of its strong hydrophobicity and consequently alter bile-acid transport. However, the basolateral membrane is not as important for bile formation as the canalicular membrane, since canalicular excretion is regarded as the rate-limiting step in the hepatic transport of bile acids [38]. In the present study, direct measurement of steady-state fluorescence anisotropy demonstrated that the fluidity of CMEVs from cyclosporin A-treated rats was lower than that of CMEVs from saline- and vehicle-treated rats. The increase in canalicular membrane rigidity induced by cyclosporin A was associated with enrichment of membrane cholesterol and a consequent increase of the cholesterol/phospholipid ratio. The mechanism by which cyclosporin A decreases membrane fluidity was not elucidated in this study. However, Backman et al. [39] have reported that cyclosporin A markedly decreased the hepatic phospholipid content in rats. Accordingly, the drug may alter phospholipid metabolism and thus increase the molar ratio of cholesterol to phospholipid. Via a reduction in membrane fluidity, cyclosporin A could reduce the activity of canalicular membrane transporters or cause a decrease in the removal of membrane lipids by bile acids.

In fact, it has been reported that an increase of membrane fluidity has a beneficial effect on cyclosporin A-induced cholestasis. Fernandez et al. [40] reported that *S*-adenosyl-L-methionine antagonized cyclosporin A-induced cholestasis in rats. Sinicrope et al. [41] demonstrated that 2-(2-methoxyethoxy)-ethyl-8-(*cis*-2-*n*-octylcyclopropyl)octanoate and benzyl alcohol increased canalicular membrane fluidity and inhibited Pgp-mediated drug accumulation in CMEVs. Furthermore, Queneau et al. [42,43] reported that tauroursodeoxycholate prevented cyclosporin A-induced cholestasis in rats. We are now investigating how different hydrophilic bile acids alter canalicular membrane fluidity and/or canalicular membrane-transporter expression.

The present study clearly demonstrates the disproportionate reduction of biliary cholesterol and phospholipid secretion when compared with that of bile acids, i.e. so-called 'uncoupling'. The mechanisms of uncoupling by cyclosporin A might include (i) the impairment of intracellular lipid transport, (ii) the selective impairment of the membrane lipid transporter (Mdr2) and (iii) the reduction of bile-acid micelle capacity to induce biliary lipid secretion. A vesicle-mediated process is involved in the transport of biliary lipid precursors to the canalicular membrane, to which

they fuse until bile acids cause the release of membrane components. Vesicles also play a role as carriers for membrane proteins. Therefore, the reduction of biliary phospholipid and cholesterol secretion may be explained by impairment of the intracellular transport of lipid-containing vesicles and/or impairment of their fusion to canalicular membrane, as occurs with phalloidin [44]. In fact, cyclosporin A has been reported to block intrahepatic vesicular transport in the rat [14]. However, there was no marked change in the expression of membrane proteins, which are also supplied by intrahepatic vesicular transport, suggesting that vesicular traffic was not reduced by cyclosporin A. The second possibility is selective impairment of the membrane lipid transporter (i.e. Mdr2). However, the present study showed no change in the expression of this transporter, although a decrease of membrane fluidity may possibly reduce the activity of membrane lipid transporters, including the phospholipid transporter Mdr2 (characterized), the cholesterol transporter Abc1 (uncharacterized). Accordingly, further investigations are needed to clarify this situation. Finally, cyclosporin A may bind to bile-acid micelles and create hybrid micelles by which lipid extraction is less easy, which we have reported as a possible explanation for the organic-anion-induced uncoupling phenomenon [45–47]. Again, the present data are not sufficient to clarify this hypothesis.

Recently, Stieger et al. [48] reported that oestradiol-17 β -glucuronide causes *trans*-inhibition of Bsep after its intracanalicular accumulation was mediated by Mrp2. This report suggests another possible action of cyclosporin A in the canalicular cavity. Because drug-induced inhibition of canalicular phospholipid secretion could result from a *trans*-effect of cyclosporin A, the direction of action should also be assessed in the future.

In summary, we have demonstrated that cyclosporin A caused uncoupling of biliary lipid secretion in rats, which was associated with an increase of the cholesterol/phospholipid molar ratio in the canalicular membrane. The fact that expression of canalicular membrane transporters was unchanged (except for Pgps) despite the marked reduction of biliary lipid secretion suggests that transport activity is at least partly dependent on canalicular membrane fluidity. Thus changes in the distribution of lipids that modulate cell-membrane fluidity may play an important role in the regulation of hepatocellular function.

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