Enrichment of canalicular membrane with cholesterol and sphingomyelin prevents bile salt-induced hepatic damage

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Abstract **These studies were undertaken to characterize the role of plasma membrane cholesterol in canalicular secretory functions and hepatocyte integrity against intravenous taurocholate administration. Cholesterol and sphingomyelin concentrations and cholesterol/phospholipid ratios were significantly increased in canalicular membranes of diosgenin-fed rats, suggesting a more resistant structure against solubilization by taurocholate. During taurocholate infusion, control rats had significantly decreased bile flow, whereas diosgenin-fed animals maintained bile flow. Maximal cholesterol output increased by 176% in diosgenin-fed rats, suggesting an increased precursor pool of biliary cholesterol in these animals. Maximal phospholipid output only increased by 43% in diosgenin-fed rats, whereas bile salt output remained at control levels. The kinetics of glutamic oxalacetic transaminase, lactic dehydrogenase, and alkaline phosphatase activities in bile showed a significantly faster release in control than in diosgenin-fed rats. After 30 min of intravenous taurocholate infusion, necrotic hepatocytes were significantly increased in control animals. Preservation of bile secretory functions and hepatocellular cytoprotection by diosgenin against the intravenous infusion of toxic doses of taurocholate was associated with an increased concentration of cholesterol and sphingomyelin in the canalicular membrane. The increase of biliary cholesterol output induced by diosgenin was correlated to the enhanced concentration of cholesterol in the canalicular membrane.**—Amigo, L., H. Mendoza, S. Zanlungo, J. F. Miquel, A. Rigotti, S. Gonzalez, and F. Nervi. **Enrichment of canalicular membrane with cholesterol and sphingomyelin prevents bile salt-induced hepatic damage.** ***J. Lipid Res.*** **1999.** 40: **533–542.**

Supplementary key words biliary lipids • cholesterol • bile salts • plasma membrane • membrane lipids • canaliculus • cytoprotection • sphingomyelin • maximal secretory rates • diosgenin

The enterohepatic circulation of bile salts (BS) is one of the principal driving forces for the secretion of biliary cholesterol, phospholipids, endobiotics, and xenobiotics, and plays a critical role in cholesterol and fat absorption (1). At increasing concentrations of plasma circulating BS, maximal secretion rates (SRm) of BS and biliary lipids are rapidly reached, and then decline with bile flow. This indicates functional damage in the secretory functions of the apical pole of the hepatocyte (1–3). Functionally, BS SRm reflect the rate-limiting step of biliary lipid output under normal conditions (4, 5). Further increase of hepatocellular uptake of BS above its SRm is followed by functional and structural liver damage (3, 6), including a decrease in bile flow, biliary cholesterol and phospholipid outputs (5). Several experimental models have shown that BS-associated liver damage would depend on the total amount and the hydrophobic–hydrophilic balance of BS species in the enterohepatic circulation, with more hydrophobic species inducing more cell damage (6–9).

BS-induced functional and structural liver damage also depends on the presence of cell membrane-associated cytoprotective factors such as cholesterol and phosphatidylcholine (7–12). BS-associated cell injury has been related to increased membrane fluidity and permeability after membrane lipid solubilization (13). Phospholipids and cholesterol provide in vitro cytoprotection against BS-induced hemolysis and CaCo-2 cell death in vitro (14). Similarly, high secretory rates of cholesterol into bile decreased BS-induced liver damage after bile duct ligation (15). The development of the mdr-2 knockout mice, who exhibited spontaneous liver damage (16, 17), confirmed a tight relationship between cholesterol and phospholipid secretion into bile and susceptibility to BS-induced cytotoxicity. Another important hypothesis that emerged from these studies was that the amount of specific lipids contained in subcellular compartments of the hepatocyte would be also key determinants for both BS-coupled lipid secretions into bile as well as prevention of BS-induced hepatocellular damage.

Neither the immediate subcellular origin nor the mech-

Abbreviations: SRm, maximal secretory rate; TC, taurocholate; SCP-2, sterol carrier protein 2; IV, intravenous; d-f, diosgenin-fed; AP, alkaline phosphatase; Na^+, K^+-ATPase, sodium potassium adenosine triphosphatase; BS, bile salts; LDH, lactic dehydrogenase; SGOT, serum glutamic oxalacetic transaminase.

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anism(s) of biliary cholesterol and phospholipid secretion have been completely elucidated. Biliary phospholipid apparently originates from vesiculation of the outer leaflet of the canalicular membrane as a consequence of the mdr-2 phosphatidylcholine flippase activity (18, 19). It has been hypothesized that biliary cholesterol also might be directly derived from some microdomains of the canalicular membrane through a bile salt-mediated solubilization process (20–22). Cholesterol could then be incorporated and carried in the lamellae of biliary phospholipid vesicles. Thus, it could be theoretically possible that the availability of membrane phospholipids and cholesterol might finally determine both the amounts of lipids for secretion into bile, as well as the preservation of the structure and secretory functions of the apical domain of hepatocytes when exposed to high concentrations of BS.

We have postulated that diosgenin-associated hepatic cytoprotection after bile duct obstruction was mainly the result of a high concentration of bile cholesterol, which could decrease the detergent effects of BS on canalicular and bile ductular apical membranes (15). However this model was unable to take into account possible changes of lipid composition of canalicular membranes that might explain cell resistance against BS solubilization in the diosgenin-fed (d-f) animal. Our current hypothesis is that the content and type of apical membrane lipid might be another limiting factor of hepatocyte resistance against the toxic effects of BS. To elucidate the role of lipid composition of liver membranes on bile secretory functions and hepatocyte structural integrity, we used the d-f rat model under high doses of intravenous taurocholate (IV TC) infusion. This manoeuver is usually deleterious for cell integrity and function in the control rat (6, 11, 12). We measured bile flow, maximal secretory rates (SRm) of BS and lipids, and biochemical as well as histological markers of hepatocellular injury in the d-f rat model.

MATERIALS AND METHODS

Materials

Diosgenin ([25R]-5-spirosten-3β-ol), taurocholic acid sodium salt (TC), NAD, citric acid, glucose-6-phosphate, AMP, DPT, UDP-d-galactose,β-galactosidase, catalase, alcohol dehydrogenase, alkaline phosphatase, Substrate Sigma104, 3α-hydroxysteroid dehydrogenase, sucrose, galactose, N-acetyl-d-glucosamine, and UDP-galactose were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium azide, EDTA, Tris-HCl, trichloroacetic acid, HPLC Lichrospher Si 100, and all solvents were obtained from E. Merck (Darmstadt, Germany). Radioactive compounds were purchased from New England Nuclear (Boston, MA), including UDP—[^{14}C]galactose (337 mCi/mmol). Peroxidase-conjugated rabbit IgG fraction to rat albumin was purchased from Cappel Research Products (Durham, NC), and pentobarbital was from Abbott Laboratories (Chicago, IL).

Animals and diets

Male Sprague-Dawley rats (200–230 g) were used in all experiments. Animals were fed a casein-based control diet (23) and were subjected to reverse light cycling for at least 2 weeks prior to the experiments. Diosgenin (0.5%, wt/wt) was dissolved in chloroform, mixed with casein-based control diet, dried under a hood, and fed to the rats for 10 days prior to the experiments. On the day of the experiments, rats were anesthetized with intraperitoneal pentobarbital (45 mg/kg-body wt) at 8–9:30 am. Bile fistula was performed as previously described (24, 25). All experiments were executed according to accepted criteria for the humane care of experimental animals.

Bile sampling

Bile was collected at 10-min intervals, for bile flow, BS, biliary lipids, albumin, and enzyme activity determinations. Sodium taurocholate dissolved in 0.9% NaCl was intravenously infused at 3 μmol $\times$ min^{-1} $\times$ 100 g body wt^{-1} in control and d-f rats. Nine bile specimens were obtained every 10 min during the experimental period. Maximal bile flow and, SRm for bile salt, phospholipid and cholesterol were calculated as the mean of the two consecutive highest secretion values. A second set of experiments was designed to determine the effect of diosgenin on the kinetics of TC-induced hepatocellular secretory failure. TC was infused at stepwise increasing doses of 1.2, 1.6, 2.4, and 3.2 μmol $\times$ min^{-1} $\times$ 100 g body wt^{-1} for 30 min each period. Bile flow, BS, phospholipid, and cholesterol outputs were measured in each bile sample.

Morphological analysis

In some experiments, fresh hepatic tissue was obtained from control and d-f rats after 60 min of taurocholate infusion (1.6 μmol/min), fixed in 10% buffered formalin, embedded in Paraplast, and cut in 5–6 μm thick sections. Specimens were stained with hematoxylin and eosin for light microscopy. Quantitative light microscopy analysis was performed on three livers of each group. Tissue specimens were obtained at 60 min of the stepwise-increasing TC infusions, while infusing 1.6 μmol·min^{-1}·100 g body wt^{-1}. Necrotic cells were identified by commonly accepted pathological criteria. Stereological analysis was performed using a systematic grid (26). Briefly, randomly selected areas were photographed and 12 $\times$ 18 cm prints were obtained. A point intersection grid drawn on a transparent film was then applied. The points falling on necrotic hepatocytes were counted and the area density was determined. Counting was performed on four periportal triads (zone 1) and four perivenous central areas (zone 3). The numbers of necrotic hepatocytes were expressed as the mean $\pm$ SD of necrotic cells per total area, in mm^2. Necrotic hepatocytes were independently counted by two of the authors. Correlation coefficient between observers was 0.95 ($P < 0.001$).

Isolation of hepatic subcellular fractions from rat liver

Liver tissue fractionations and marker enzymes were performed as previously described (22). Nuclear, mitochondrial, microsomal, and supernatant fractions were isolated as described by de Duve et al. (27) and Bronfman et al. (28). Isolated fractions were resuspended in 0.25 m sucrose containing 3 mm imidazole, pH 7.4. Golgi apparatus membranes were obtained and characterized (29, 30). This fraction was enriched 72-fold in galactosyltransferase activity. These Golgi membrane preparations were essentially free of plasma membrane, light mitochondrial fraction, and endoplasmic reticulum contamination, as measured by activities of 5′-nucleotidase and aminopeptidase-N, acid phosphatase, and glucose-6-phosphatase, respectively.

Basolateral and canalicular membrane separation was performed following the method of Rosario et al. (31). Briefly, liver slices were homogenized with a Polytron apparatus (Kinematica GmbH, Littau, Switzerland) in chilled buffer containing 300 mm mannitol, 18 mm Tris-HCl, pH 7.4, 5 mm ethylene glycol bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, and 0.1 mm phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 49,000 *g* for 35 min. The pellet was resuspended and precipitated

with 15 mm $MgCl_2$. The supernatant of the $MgCl_2$ precipitation was centrifuged at 49,000 *g* for 35 min to obtain canalicular membranes. The initial pellet from the $MgCl_2$ precipitation was resuspended in 50% sucrose, overlaid on a 41%/38% discontinuous sucrose gradient, and centrifuged at 88,000 *g* for 3 h. The sinusoidal membranes-containing top layer was harvested. Both canalicular and sinusoidal membrane fractions were resuspended in 1 mm $NaHCO_3$ and stored at −20°C. Purity of fractions was assessed by common enzymatic techniques.

Quantitative inmunoblotting, chemical, and enzymatic analysis

Total proteins were measured by the Lowry method (32). Phospholipids, cholesterol and BS were quantified as previously described (23–25). HPLC was used to quantify the content of phosphatidylcholine and sphingomyelin in membrane fractions (15, 33). The biliary levels of common marker enzymes of liver damage (SGOT, LDH, and AP) were tested by conventional automated techniques. Biliary albumin was measured by quantitative immunoblotting, using a rabbit polyclonal antibody (34). Aminopeptidase-N activity was assessed by a fluorimetric method (35). Marker enzymes of cellular organelles were determined as previously described (27, 28). Galactosyltransferase activity was also determined in Golgi-rich membrane preparations (29). Na^+, K^+-ATPase activity was measured after freeze–thawing by the method of Ismail-Beigi and Edelman (36).

Calculations, presentation, and statistics of the data

Unesterified and esterified cholesterol distribution in subcellular fractions is shown as normalized and averaged histograms (28). The ordinate represented the relative specific activity of cholesterol in each subcellular fraction: $[Q/\Sigma Q \cdot \Delta\delta]$, where Q represents the cholesterol concentration of the fraction, ΣQ the total cholesterol content and $\Delta\delta$ the relative increment in protein content of subcellular fractions. Relative specific activity of cholesterol was plotted against the relative increment of hepatic protein in the subcellular fractions. The kinetics of bile flow and BS, cholesterol, and phospholipid secretion into bile were studied as a function of time. Biliary BS secretion rates reached a maximum and then declined together with bile flow and biliary lipids. The best mathematical fitting for each parameter was a non-linear cubical regression of the form y = a + bx + cx2 + dx3. In this equation, y represents the measured parameter in bile as a function of time (x). The letters a, b, and c are constants. Non-linear cubic curves derived for each experimental parameter had a good and statistically significant fitting with a proportion of variance between 72% and 97% in all series of experiments. Data of curves represent the mean values for each period of bile collection during the experiments. SE varied between 8% to 24% of the mean in all calculated data presented in the figures. Significant differences between curves were calculated by likelihood ratio tests. Student's unpaired *t* test or one-way analysis of variance was used for data analysis of some experiments. Statistics were performed with software SPSS 7.5 for Windows.

RESULTS

Lipid composition of hepatic subcellular fractions

The subcellular distribution of hepatic proteins as well as free and esterified cholesterol was similar in control and d-f animals, as shown in **Table 1**. The relative subcellular distribution of these constituent compounds presented a normal pattern in the diosgenin group (**Fig. 1**), with the exception of the increased percentage of cholesteryl esters found in the light mitochondrial fraction (14% versus 5.7% in control animals, $P < 0.01$). The measurements of succinic dehydrogenase, reduced nicotinamide adenine-dehydrogenase, and N-acetyl-glucosaminidase activities indicated minimal contamination of each subcellular fraction (results not shown). The most important finding of these series of experiments is shown in **Table 2**. Diosgenin feeding did not alter the isolation and enrichment of plasma membrane fractions, as recovery and enrichment of AP and Na^+, K^+-ATPase specific activities were similar in both groups of animals (results not shown). As expected (31, 37), Na^+, K^+-ATPase specific activities were very low in the canalicular plasma membrane fraction in both groups of rats (Table 2), indicating no significant contamination of this fraction by sinusoidal plasma membranes. As also shown in Table 2, AP specific activities were very low in sinusoidal plasma membrane fractions, also indicating no significant contamination by canalicular membrane fractions in control and d-f animals. In the diosgenin group, cholesterol, total phospholipid, and sphingomyelin concentrations of canalicular membranes were significantly increased by 28%, 8%, and 34%, respectively. The cholesterol/phospholipid ratio of sinusoidal membranes significantly in-

TABLE 1. Subcellular fractionation of rat liver homogenates by differential centrifugation

	Distribution in Subcellular Fractions					
			Cholesterol			
	Protein		Free		Esters	
Fractions	C	D	C	D	C	D
	%		%		%	
Nuclear	13 ± 0.6	15 ± 0.8	25 ± 1.5	22 ± 2.4	11 ± 0.5	8 ± 0.5
Mitochondrial						
Heavy	25 ± 1.2	24 ± 0.6	21 ± 0.4	19 ± 2.3	32 ± 4.1	31 ± 5.2
Light	2.9 ± 0.2	4.7 ± 0.4	3.7 ± 0.4	5.3 ± 0.3	5.7 ± 0.7	14 ± 3.1[a]
Microsomal	17 ± 1.4	16 ± 0.8	30 ± 0.6	24 ± 3.5	30 ± 5.2	24 ± 2.9
Supernatant	43 ± 1.6	41 ± 0.7	20 ± 2.4	30 ± 8.0	21 ± 2.2	22 ± 4.1

Values are means ± SD of four rats in control (C) and diosgenin (D) groups. Protein and cholesterol concentrations in homogenates were: Protein C = 208 ± 19, D = 215 ± 23 m × g^{-1}; Free cholesterol: C = 2.1 ± 0.8, D = 1.9 ± 0.3 mg × g^{-1}; Cholesteryl esters: C = 0.20 ± 0.05, D = 19 ± 0.2 mg × g^{-1}.

[a] Significant difference at $P < 0.01$.

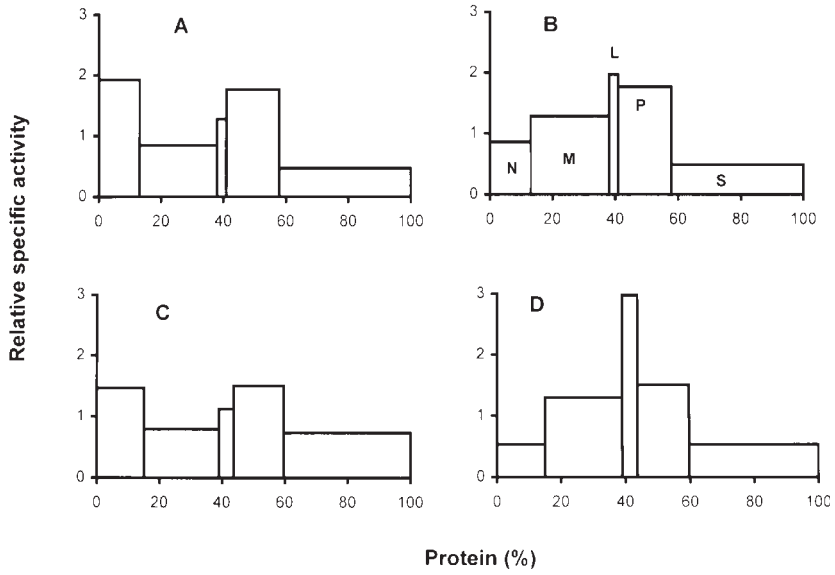

Fig. 1. Subcellular cholesterol distribution in fractions of rat liver homogenates obtained by differential centrifugation. The histograms represent the mean of four liver fractionations in each group. Panels A and B represent the free and ester cholesterol species of control rats, respectively. Panels C and D represent the diosgenin group. Letters of panel B indicate the sub-cellular fraction: N, nuclear fraction; M, mitochondrial heavy fraction; L, mitochondrial light fraction (it contains lysosomes); P, microsomal fraction; S, supernatant fraction. *Only cholesteryl esters of fraction L were significantly increased in d-f animals* ($P < 0.01$).

creased from 0.17 to 0.21 ($P < 0.01$) and that of canalicular membranes from 0.33 to 0.44 ($P < 0.01$) in diosgenin-treated rats. The relative contribution of sphingomyelin to total phospholipids of canalicular membranes significantly increased from 15% to 18% in the diosgenin group ($P < 0.01$), whereas this species only represented 3% of the total phospholipids of the sinusoidal membranes in both groups of animals. Purity of all these membrane fractions was assessed by AP specific activities. Similarly, cholesterol concentration of Golgi membranes was within the normal range in diosgenin-fed rats (results not shown). Taken together, these cell fractionation studies indicated that diosgenin induced important quantitative and qualitative lipid changes in liver cell membrane fractions, particularly at the apical pole of hepatocytes.

Effect of diosgenin feeding on bile flow, BS, and lipid SRm induced by TC

As expected, diosgenin feeding enhanced biliary cholesterol output with a significant increase of 300%, from 0.44 ± 0.03 to 1.45 ± 0.32 nmol $\times$ min^{-1} $\times$ g^{-1}. All other basal parameters of biliary lipids, BS secretion, and bile flow remained within the normal range in d-f rats, as shown in **Fig. 2** and **Fig. 3**. Basal bile flows were similar in both groups. Bile flow approximately doubled in control and d-f rats after 20–30 min of TC intravenous infusion at 3 mmol $\times$ 100 g^{-1} body wt (panel A of Fig. 2). Control rats had significantly decreased bile flow after 50 min of TC infusion, whereas at the end of the experiments the d-f group still had a bile flow similar to pre-TC infusion levels. As shown in panel B of Fig. 2, a similar pattern was observed with the kinetics of BS secretion, with a more rapid decrease of BS output after 30 min of IV TC infusion in the control rats. The calculated kinetics of bile flow and BS output as a function of increasing IV infusions of TC were significantly different in d-f rats, as assessed by likelihood ratio tests. However the SRm of BS outputs were similar in the two groups of animals (**Table 3**).

As shown in Table 3, SRm of both phospholipid and cholesterol into bile significantly increased by 43% and 176%, respectively, in the diosgenin group of rats. In spite of higher SRm of phospholipids. The kinetics of both phospholipid and cholesterol outputs were different under TC infusion in control compared to d-f animals (Fig. 3). The

TABLE 2. Effect of diosgenin on lipid composition and enzyme marker activities of liver plasma membrane fractions

	Membrane Lipids			Marker Enzymes	
Fractions	Cholesterol	Phospholipid	Sphingomyelin	Na^+,K^+-ATPase	Alkaline Phosphatase
	nmol $\times$ *mg*$^{-1}$ *protein*			*U* $\times$ *mg*$^{-1}$ *protein*	
Sinusoidal					
Control	145 ± 14	879 ± 17	25 ± 5	20.5 ± 1.8	1.4 ± 0.4
Diosgenin	172 ± 13	848 ± 78	25 ± 6	14.4 ± 3.0	1.2 ± 0.2
Canalicular					
Control	307 ± 36	835 ± 66	125 ± 2	2.6 ± 1.4	15.7 ± 1.9
Diosgenin	392 ± 12[a]	900 ± 71[a]	167 ± 3[a]	2.1 ± 0.4	14.0 ± 2.0

Lipid concentration of plasma membrane fractions is the mean ± SD of 9 control and 6 d-f rats. Sphingomyelin concentration as quantified in 5 animals of each group. Marker enzyme activities are the mean ± SD of 3 consecutive fractionation studies in each group.

[a] Significantly different compared to control rats at $P < 0.01$.

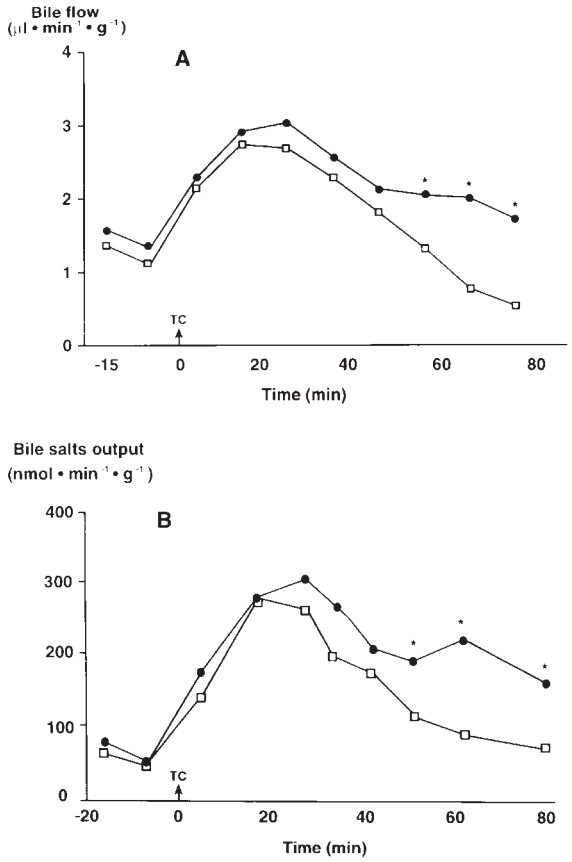

Fig. 2. Effect of diosgenin treatment on bile flow (panel A) and bile salt output (panel B) into bile. A series of four rats were IV infused with TC (3 μmole·min^{-1}·100 g body wt^{-1}) for 90 min. Rectal temperature was kept at 37°C by means of an electric lamp throughout the experiments. Bile was collected in pre-weighed tubes every 10 min. The black dots correspond to the diosgenin group and the white squares to the control animals. The calculated proportion of variances were in the range of 72% to 97% indicating a good fitting to a non-linear cubic model $y = a + bx + cx^2 + dx^3$. Likely ratio tests indicated that experimental curves were significantly different between groups in both parameters. The asterisks indicate a significant difference in the bile collection period at $P < 0.01$.

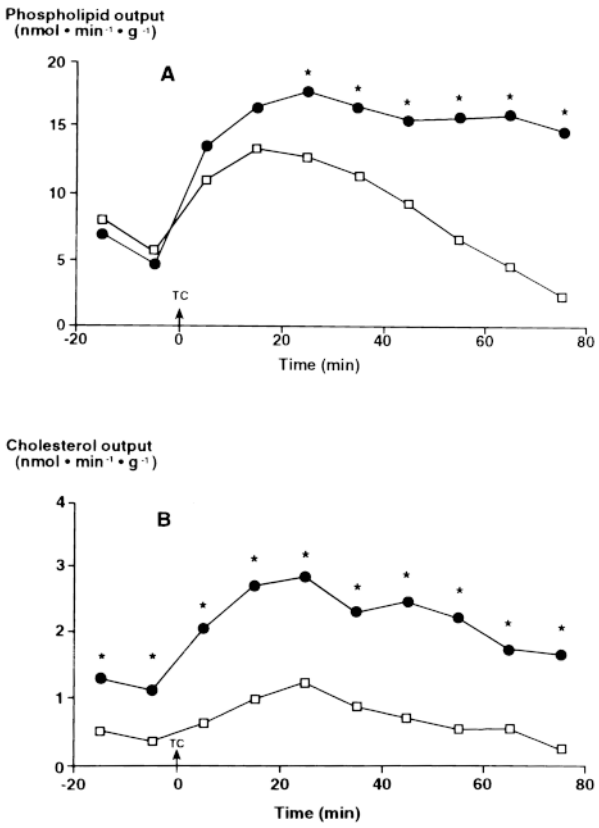

Fig. 3. Biliary phospholipid (panel A) and cholesterol (panel B) outputs. The experimental conditions are described in Fig. 1. The non-linear cubic model fitted the experimental data. Likelihood ratio tests indicated that the curves of the diosgenin group were significantly different. Asterisks indicate a significant difference ($P < 0.01$) in the corresponding period of bile collection.

kinetics of both lipids presented a similar pattern with a progressive decrease of lipid outputs after reaching SRm at approximately the same time (25–40 min) in control animals. In contrast, d-f rats maintained significantly higher phospholipid and cholesterol outputs throughout the TC-infusion experiments. Biliary lipid secretory values were significantly increased at the end of the infusions of TC compared to the basal outputs.

Effect of diosgenin on TC-induced hepatocellular damage

To determine the effect of intravenous TC on biochemical and morphological hepatocellular damage, progressively higher amounts of TC were intravenously infused into rats, as described in Materials and Methods. In addition, albumin secretion into bile as a function of time was measured to assess tight junction permeability (12). The time course of release of intracellular and membrane-associated enzymes into bile is shown in **Fig. 4** and **Fig. 5**.

The kinetics of intracellular enzyme release were significantly different in each group of animals, with a faster increase of biliary SGOT and LDH activities in the control rats (Fig. 4). Biliary aminopeptidase-N, a canalicular amphiphilic ectoenzyme, was released into bile in significantly higher amounts in control animals after 30 min of TC infusion, whereas in d-f rats the biliary enzyme activity remained essentially within the normal range throughout

TABLE 3. Effect of diosgenin on maximum bile flow, bile salt and lipid secretion rates (SRm)

Group	n	Bile Flow	Maximum Secretory Rates		
			Bile Salts	Phospholipids	Cholesterol
		$\mu l \times min^{-1} \times g^{-1}$	$nmol \times min^{-1}1^{1} \times g^{-1}$		
Control	4	2.7 ± 0.2	271 ± 18	11.5 ± 1.1	1.12 ± 0.14
Diosgenin	4	2.9 ± 0.3	299 ± 32	16.0 ± 1.4[a]	3.10 ± 0.62[a]

Rats were intravenously infused with 3 μmol × min^{-1} taurocholate for 90–120 min. Maximum bile flow, bile salt and lipid rates were estimated as the mean value of the two higher values obtained during the experimental period. Maximum bile flow and bile salt SRm were obtained at 20–30 min of infusion in both groups.

[a] Significant difference at $P < 0.01$.

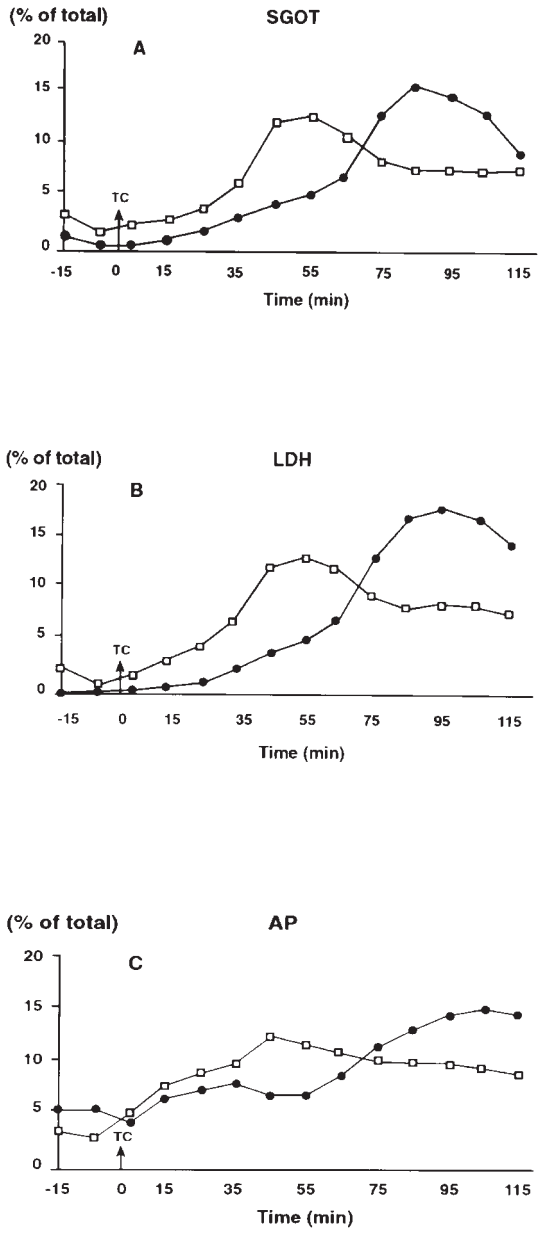

Fig. 4. Effect of diosgenin on biliary SGOT, LDH, and AP as a function of stepwise increasing doses of IV TC infusions. Each dose was infused for 30 min: 1.2, 1.6, 2.4, and 3.2 μmol·min^{-1}·100 g body wt^{-1} and bile aliquots were collected every 10 min in pre-weighted tubes. Each point of the curves represents the mean of four animals. Non-linear cubic curves were derived from the experimental data. There was a good fitting indicated by significantly high calculated proportions of variances (>84%) in all experimental groups. The likelihood ratio tests indicated that all curves of d-f rats were significantly different compared to control animals.

the experiments (Fig. 5). The kinetics of biliary albumin output were in a similar range in both groups of animals, suggesting that the tight junctions were functionally preserved during the TC infusion experiments in control and d-f rats (results not shown). At the time when enzyme release into bile began to increase (after 60 min of IV TC infusion), liver specimens were obtained for quantitative histology from three animals of each experimental group.

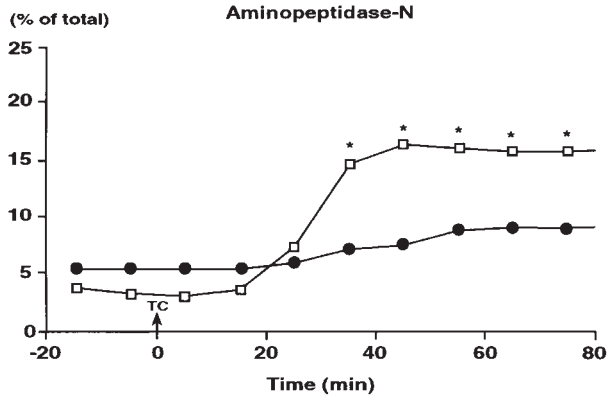

Fig. 5. Effect of diosgenin on biliary aminopeptidase-N activity during IV TC infusion. The experimental conditions were those described in Fig. 2. The asterisks indicate a significant difference at $P < 0.01$.

Necrosis was more evident in the control than in d-f rats, as shown in panel B of **Fig. 6**. The number of periportal (zone 1) necrotic hepatocytes was 3.5 ± 0.3 and 1.1 ± 0.07 per mm^2 in control and d-f rats, respectively ($P < 0.001$). In central venous areas (zone 3), the control animals also presented a higher number of necrotic cells compared to the diosgenin group of rats: 4.3 ± 0.21 vs. 0.75 ± 0.004 per mm^2 ($P < 0.001$). These observations showed that there was a good correlation between the rise of biliary levels of hepatocellular enzyme and the number of necrotic cells.

DISCUSSION

The results of these experiments were consistent with the working hypothesis of this study. The d-f rat had significant changes in the lipid composition of plasma membranes, particularly of the canalicular membrane. The enhanced cholesterol and phospholipid content of the canalicular membrane of the diosgenin group paralleled both, the marked output of cholesterol into bile as well as the increase of SRm of cholesterol and phospholipids. Another important finding was the in vivo protective capacity of diosgenin against bile salt-associated hepatocellular damage and secretory failure, a phenomenon correlated also to the increase of cholesterol and sphingomyelin content in the canalicular membrane. This result was also consistent with previous in vitro studies, showing a protective role of cholesterol and phospholipids against the detergency of cell membranes by BS (7–14).

It is generally accepted that biliary lipids have important physiologic functions. These include the elimination of sterol molecules from the organism as cholesterol and BS to maintain body cholesterol homeostasis, the formation of biliary vesicles and mixed micelles to facilitate the excretion of amphiphilic and hydrophobic endo- and xenobiotics, and the promotion of lipid digestion and absorption in the intestine (8–11). As expected, feeding diosgenin massively increased basal biliary cholesterol output, without significant changes of bile flow, phospho-

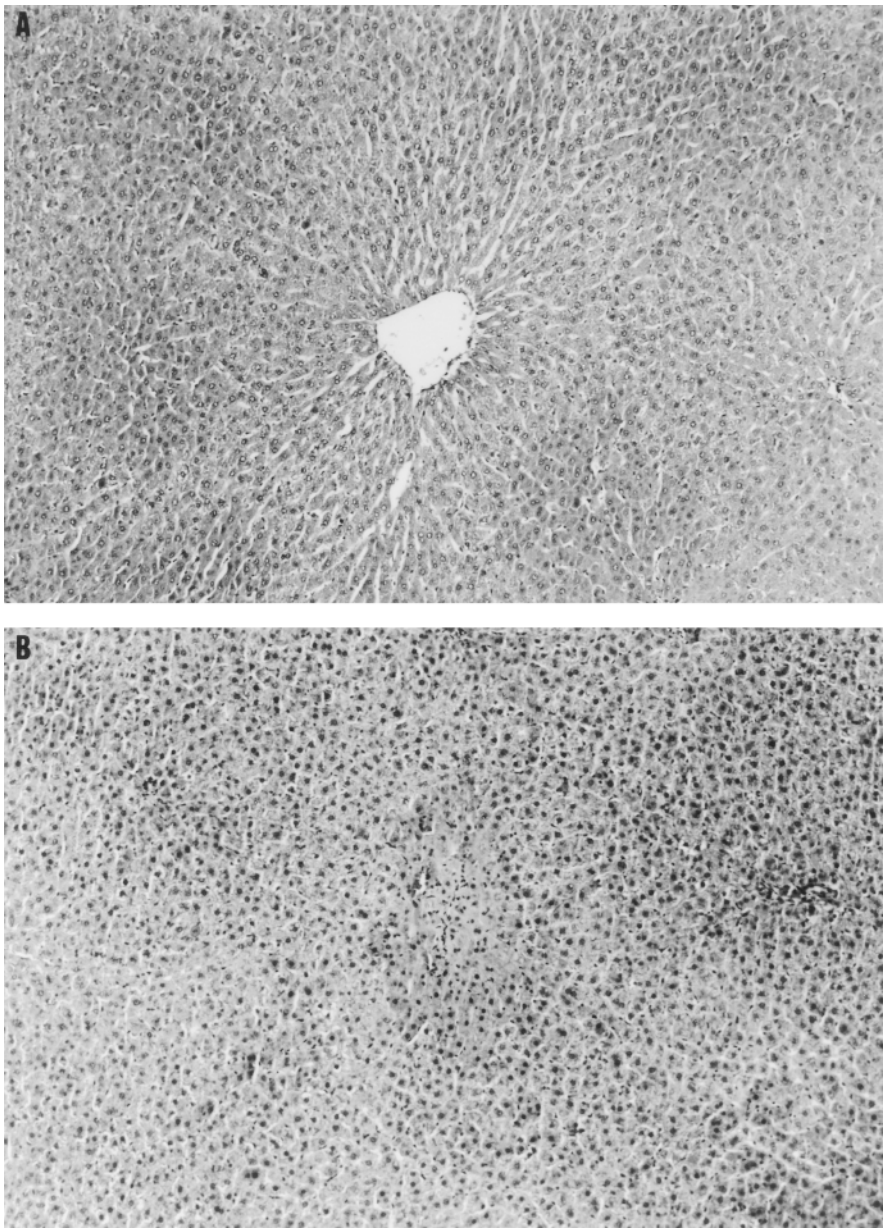

Fig. 6. Light photomicrographs of rat liver tissue subjected to TC infusion. Tissue specimens were obtained at 60 min of the stepwise-increasing TC infusions, while infusing 1.6 μmol·min^{-1}·100 g body wt^{-1}. (A), d-f rat and (B), control rat. Dark nuclei represent damaged cells, which are more abundant in control rats as seen in panel B.

lipid and BS secretion into bile (25). We found that BS-induced bile secretory failure was prevented by diosgenin, as assessed by the measurement of bile flow, phospholipid, bile salt, and cholesterol outputs under high IV infusions of TC. Interestingly, SRm of phospholipid and cholesterol, besides being significantly increased, remained over pre-infusion basal rates, as also occurred with BS output. This finding was consistent with the hypothesis that diosgenin feeding increased the precursor pools of biliary lipids, in the canalicular membrane prior to its secretion into bile. Biochemical and morphological criteria clearly indicated that the experimental maneuver was successful in preventing hepatocyte damage. This effect was probably also the result of the higher lipid content of plasma membranes (specifically, sphingomyelin and cholesterol in the canalicular membrane). These molecules have been found to decrease membrane fluidity (38, 39) and to increase its resistance to detergent solubilization. The purity of sinusoidal and canalicular membrane fractions was assessed by the enrichment of specific enzyme markers. Enrichment of Na^+, K^+ ATPase and AP specific activities were used as markers of the sinusoidal and canalicular plasma membrane domains, respectively. The degree of membrane purification obtained in these experiments was in the range of previously published studies (31, 37).

Three important observations of these studies should be remarked. First, contrarywise to a previous study in which there was no modification in the subcellular cholesterol distribution in the liver of d-f rats (40), we consistently found, using a different method of organelle separation, that diosgenin feeding increased the concentrations of cholesterol in the lysosomal compartment. Most intracellular organelles contain little cholesterol, with the exception of lysosomes and Golgi (31, 41, 42). The increased content of cholesterol found in the lysosomal fraction of d-f rats is consistent with the enhanced hepatic uptake of lipoprotein cholesterol in d-f rats, as previously shown in this laboratory (43). In addition, the cholesterol/phospholipid ratio was increased in both basolateral and canalicular plasma membrane fractions. Although we only estimated crude concentration values, the simultaneous increase of cholesterol and sphingomyelin in canalicular membranes of d-f rats indirectly suggested the presence of a more rigid and resistant membrane structure (38) in the biliary secretory apparatus. This finding was consistent with the preservation of the secretory functions at the apical pole of hepatocytes when exposed to toxic IV infusions of TC. The biliary secretory apparatus of d-f animals produced almost normal levels of bile flow, bile salt and lipid secretion under the high amounts of IV TC being transported through the efficient BS secretory pathway. In contrast, control rats had secretory failure and hepatocellular damage.

Second, the enhanced concentrations of cholesterol and phospholipid in the canalicular membrane of d-f rats were correlated with increased basal biliary cholesterol secretion, as well as increased SRm of both cholesterol and phospholipid into bile. The finding of enhanced concentrations of cholesterol and phosphatidylcholine in canalicular membranes of d-f rats is consistent with the hypothesis that the immediate precursor pools of both biliary lipids are localized in some subcompartment (s) of that membrane (1–3). This interpretation is supported by the fact that approximately 70–90% of free cholesterol of cells, including hepatocytes, is in the plasma membrane (44, 45), which in fact represents the principal metabolically active compartment of cholesterol in the cell (38, 41, 46). Plasma membrane cholesterol is apparently distributed in two distinctive pools (41). This distribution may also apply to the canalicular membrane. One pool might be associated to sphingomyelin–cholesterol rafts, giving support to a rigid and resistant structure of the apical membrane, and a second more dynamic pool might be localized in the fluid phosphatidylcholine-rich regions of the plasma membrane (47, 48). This would represent the precursor pool from which a fraction of biliary cholesterol might originate. In non-hepatic cells, cholesterol is associated, in part, with specialized areas of the plasma membrane including caveolae and clathrin-coated pits, structures that have been associated with the regulation of cholesterol efflux from cells (49). However, caveolae and the caveolae-associated caveolin family proteins have not yet been described in hepatocyte plasma membranes (47–49). It is unknown whether similar structures or other specific functional membrane domains in the canalicular membrane might be responsible for a bile salt-facilitated cholesterol efflux into biliary phospholipid-rich vesicles. Another key quantitative factor determining the absolute amount of lipid secreted into bile might be the SCP-2-mediated flux of intracellular cholesterol to the plasma membrane (50) and the hydrophobicity of the BS pool (51). The higher availability of cholesterol at the apical pole of hepatocytes of d-f rats as compared to control rats was presumably the result of both, an enhanced rate of hepatic cholesterogenesis and SCP-2 transport to the plasma membrane (52) and an increase of the sinusoidal uptake of lipoprotein cholesterol. In fact, lipoprotein-derived biliary cholesterol was markedly increased in d-f rats (25), a phenomenon also found in bean-fed animals (23). This observation is consistent with the enhanced sinusoidal uptake of high and low density lipoprotein cholesterol in rats with high biliary cholesterol output (43). The enhanced SRm of phospholipids in d-f rats might be associated with overexpression of the mdr-2 canalicular flippase. However, the findings of a normal basal biliary phospholipid output and levels of expression of the mdr glycoprotein family in canalicular fractions of d-f rats (unpublished results from this laboratory) are against this hypothesis. More likely the enhanced SRm of phospholipid was only the result of an increased availability of phospholipids in the biliary precursor pool of the d-f animal.

Finally, the present biochemical and morphological observations support the hypothesis that other plant sapogenins that increase biliary cholesterol output, such as saponins present in legumes (53), could also have cytoprotective activities. Feeding bean sapogenols to rats increased biliary cholesterol output >200% (54). Interestingly, chronic legume feeding also increased biliary cholesterol saturation (55) and secretion (56) in humans. These observations might imply that sapogenins present in foodstuffs represent common steroid xenobiotics that might contribute to protection of the canalicular apparatus against toxic endobiotics and xenobiotics normally excreted into bile.

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