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Preventive effect of silymarin against taurolithocholate-induced cholestasis in the rat

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Abstract

Increased amounts of monohydroxylated bile salts (BS) have been found in neonatal cholestasis, parenteral nutrition-induced cholestasis and Byler's disease, among others. We analyzed whether the hepatoprotector silymarin (SIL), administered i.p. at the dose of 100 mg/kg/day for 5 days, prevents the cholestatic effect induced by a single injection of the model monohydroxylated BS taurolithocholate (TLC, 30 μmol/kg, i.v.) in male Wistar rats. TLC, administered alone, reduced bile flow, total BS output, and biliary output of glutathione and HCO_3^- during the peak of cholestasis (−75, −67, −81, and −80%, respectively, $P < 0.05$). SIL prevented partially these alterations, so that the drops of these parameters induced by TLC were of only −41, −25, −60, and −64%, respectively ($P < 0.05$ vs. TLC alone); these differences between control and SIL-treated animals were maintained throughout the whole (120 min) experimental period. Pharmacokinetic studies showed that TLC decreased the intrinsic fractional constant rate for the canalicular transport of both sulfobromophthalein and the radioactive BS [^{14}C]taurocholate by 60 and 68%, respectively ($P < 0.05$), and these decreases were fully and partially prevented by SIL, respectively. SIL increased the hepatic capability to clear out exogenously administered TLC by improving its own biliary excretion (+104%, $P < 0.01$), and by accelerating the formation of its non-cholestatic metabolite, tauromurideoxycholate (+70%, $P < 0.05$). We conclude that SIL counteracts TLC-induced cholestasis by preventing the impairment in both the BS-dependent and -independent fractions of the bile flow. The possible mechanism/s involved in this beneficial effect will be discussed.

Keywords: Bile salt-dependent bile flow; Bile salt-independent bile flow; Cholestasis; Phase I metabolism; Silymarin; Taurolithocholate

1. Introduction

Lithocholate (LC) is a secondary, monohydroxylated BS formed by bacterial dehydroxylation of chenodeoxycholate (CDC). Although LC and its amide conjugates usually represent less than 5% of the endogenous BS pool [1,2], due to their poor enterohepatic circulation [3], their levels could increase in several hepatic pathologies where they may either initiate or perpetuate hepatic failure. Indeed, the taurine conjugate of LC, TLC, and other monohydroxylated BS were suggested to play a role in liver dysfunction occurring in primary biliary cirrhosis [4], Byler's disease [5], total parenteral nutrition-induced cholestasis [6], and neonatal cholestasis [7].

TLC-induced cholestasis has also been shown to be a useful experimental model of drug-induced cholestasis [8–10]. TLC induces an acute, reversible cholestasis in the rat [8], with a bile flow (BF) nadir at 15–20 min after its administration. Following this insult, BF recovers slowly, reaching control values at approximately 24–30 hr [8]. The mechanisms by which monohydroxylated BS induce cholestasis are far from being elucidated. TLC diminishes both the BS-independent (BSIF) and the BS-dependent (BSDF) fractions of the BF. The diminution in BSIF has been proposed to be due to a reduction in the water permeability

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Abbreviations: BS, bile salt/s; SIL, silymarin; TLC, taurolithocholate; LC, lithocholate; CDC, chenodeoxycholate; BF, bile flow; BSIF, bile salt-independent bile flow; BSDF, bile salt-dependent bile flow; GSH, glutathione; α-MC, α-muricholate; β-MC, β-muricholate; MDC, murideoxycholate; [^{14}C]TC, [^{14}C]taurocholate; BSP, sulfobromophthalein; TMDC, tauromurideoxycholate.

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of the canalicular membrane, as a consequence of an increment in the cholesterol:phospholipid ratio [11]. An impairment in biliary excretion of glutathione (GSH) has also been postulated to be involved [12]; since canalicular Mrp2 was characterized as a putative GSH transporter [13], its endocytic internalization occurring in TLC-induced cholestasis [14] could contribute to GSH excretory failure. Although the mechanisms by which TLC impairs the BSDF are poorly understood, we recently found that TLC diminishes the biliary excretion of BS by affecting preferentially their canalicular, Bsep-mediated transport; this phenomenon was associated with internalization of Bsep into submembranous, vesicle-like structures [15].

SIL is a purified extract from the milk thistle *Silybum marianum* (L.) Gaertn, and is composed of a mixture of four isomeric flavonolignans, namely: silibinin, isosilibinin, silidianin, and silichristin [16]. Extracts of milk thistle have been used as medical remedies for almost 2000 years, and continue to be used as therapeutic agents for many types of acute and chronic liver diseases (for reviews, see refs. [17,18]). In addition, SIL was shown to protect experimental animals from various hepatotoxicants, including carbon tetrachloride and acetaminophen, among others [19]. The mechanisms by which SIL exerts its hepatoprotective action appears to be multifactorial. SIL stabilizes membranes, thus preventing hypotonicity- and detergent-induced lysis [20]. SIL has free radical scavenger properties, thus preventing membrane lipid peroxidation in liver cells [21]. SIL exhibits antifibrotic properties, probably by blocking proliferation of hepatic stellate cells [22] and by inhibiting function of Kupffer cells [23], two cell lines involved in fibrogenesis. Finally, this flavonolignan was shown to be a potent protein synthesis inducer *via* stimulation of ribosomal RNA synthesis, by activation of polymerase I [24].

We have recently shown that SIL prevents estrogen-induced cholestasis in the rat, both *in vivo* and in isolated hepatocyte couplets [25]. This anticholestatic effect was envisaged from previous results from our group [26], which showed that SIL expands the size of the endogenous BS pool due, in part, to an increase in α-muricholate (α-MC), β-muricholate (β-MC) and ursodeoxycholate, three BSs with similar hepatoprotective properties [27]. These BSs have been shown to have beneficial effects in both estrogen-induced cholestasis [28,29] and TLC-induced cholestasis [30,31]. Moreover, SIL induces enrichment of BS pool in MCs by stimulating 6β-hydroxylase activity, which catalyzes the formation of this BS from CDC [26]. Interestingly, LC was shown to be preferentially metabolized *via* 6β-hydroxylation, leading to formation of β-MC (3α,6β,7β-trihydroxy-5β-cholanoate) and murideoxycholate (MDC, 3α,6β-dihydroxy-5β-cholanoate) [32,33]; hydroxylation to less toxic, di- and trihydroxylated metabolites is thought to play a crucial role in the recovery from cholestasis [34].

Accordingly, the aim of the present study was to analyze the capability of SIL pretreatment to prevent the acute cholestasis induced by a single dose of TLC in the rat, focusing our attention on the possible mechanism(s) involved.

2. Material and methods

2.1. Chemicals

SIL, TLC, sulfobromophthalein (BSP), [^{14}C]taurocholate ([^{14}C]TC), 3α-hydroxysteroid dehydrogenase, 5,5′-dithiobis(2-nitrobenzoic acid), NADPH, NADP, GSH, and GSH reductase were purchased from Sigma Chemical Co. All the other reagents were of the highest analytical grade available from commercial sources.

2.2. Animals

Adult male Wistar rats weighing 300–350 g were used throughout. Before the experiments, the animals were maintained on a standard diet and water ad libitum, and housed in a temperature- (21–23°) and humidity- (45–50%) controlled room under a constant 12 hr light–dark cycle. All animals received humane care according to the criteria outlined in the "*Guide for the Care and Use of Laboratory Animals*" prepared by the National Academy of Sciences, and published by the NIH (publication 86–23, revised 1985).

2.3. Surgical procedures

Experiments were started between 09:00 and 11:00 hr to minimize influence of circadian variations. Animals were anesthetized with sodium pentobarbital (50 mg/kg body wt, intraperitoneally), and anesthesia maintained throughout. Femoral artery and vein were catheterized with PC-50 polyethylene tubings, and the common bile duct with a PE-10 polyethylene catheter (Intramedic, Clay Adams). To prevent hypothermic alterations of BF, body temperature was maintained at 37.5–38.0° with a heating lamp.

2.4. Treatments and experimental procedures

Animals were randomly divided into two experimental groups, namely:

(i) Rats receiving only the SIL vehicle (propylene glycol);
(ii) Rats which were administered daily with SIL at the dose of 100 mg/kg body wt, intraperitoneally, for 5 consecutive days.

Rats from both experimental groups were subdivided as follows:

(1) *Control group*: Rats belonging to the group (i) receiving intravenously the TLC vehicle (10% BSA in saline).

(2) *SIL group*: Rats belonging to the group (ii) receiving intravenously the TLC vehicle.
(3) *TLC group*: Rats belonging to the group (i) receiving a single, intravenous dose of TLC (3 μmol/100 g body wt, in 10% BSA in saline), administered over a 1-min interval.
(4) *SIL + TLC group*: Rats belonging to the group (ii) receiving TLC as indicated above.

The TLC pulse was given after 30 min of basal bile collection. Following TLC injection, bile collection was then carried out in 20-min periods for 2 hr. Next, animals were killed by exsanguination, and livers were removed and weighed.

2.5. Hepatic handling of [^{14}C]TC and BSP

The status of the BS transport systems was evaluated by analyzing the time-course of plasma decay and biliary output of the model, radioactive BS, [^{14}C]TC. For this purpose, [^{14}C]TC was administered 100 min after TLC injection, as an intravenous pulse (0.25 μCi/100 g body wt). Blood samples were collected every 1–3 min for 20 min, and bile samples every 10 min for 60 min. Then, animals were killed by exsanguination, and livers were removed and weighed.

Rats injected with BSP were used to evaluate the status of the non-BS organic anion transport systems, by analyzing the time-course of plasma decay and biliary output of the dye. For this purpose, a pulse, intravenous administration of BSP (6 mg/100 g body wt) was carried out 100 min after TLC administration, and serial blood samples were collected at 1- to 10-min intervals over the next 30 min. Bile samples were also collected in 10-min intervals over the next 60-min period [35].

In vivo, pharmacokinetic analyses of the hepatic handling of both [^{14}C]TC and BSP were carried out by plotting the plasma concentration data against time, and further fitting these curves to a biexponential equation by a least squares method; preliminary triexponential fits failed to show any improvement in the goodness of fit, as judged by the Akaike's criterion [36]. Therefore, a two-compartmental model with an open-ended biliary out-flow, as described by Richards *et al*. [37], was considered physiologically realistic for compartmental analysis. The fractional transfer rates for the transport of the markers from plasma to liver (hepatic uptake: r_{12}), liver to plasma (sinusoidal efflux: r_{21}) and liver to bile (canalicular excretion: r_3) were calculated, as previously described [37].

2.6. Analytical procedures

BF was determined by gravimetry, assuming a bile density of 1.0 g/mL. The biliary excretion rate was calculated as the product between BF and biliary concentration. Bile was assayed for both total and individual BS compositions. Total BSs were assayed by using the 3α-hydroxysteroid dehydrogenase procedure [38]. Individual BS were determined by HPLC (Waters), as reported previously [39], and identified using appropriate standards. Since conjugates of cholate are not metabolic products of TLC, the output of cholate conjugates was employed to discriminate changes in endogenous secretion of any BS from those produced by the excretion of the exogenously administered TLC and its metabolites, as described previously [32].

To determine biliary HCO_3^- secretion, bile was collected under liquid Vaseline™ in pre-weighed tubes. HCO_3^- concentrations were calculated from pH and pCO_2 using the Henderson–Hasselbalch equilibrium equation. pH and pCO_2 were measured immediately after bile collection in an automated blood-gas analyzer (Compact 1, AVL Medical Instruments AG).

GSH was determined in bile collected in pre-weighed tubes containing 1 mL of 6% 5-sulfosalicylic acid. Subsequently, bile samples were centrifuged and total biliary GSH content (reduced plus oxidized forms) was measured in the supernatant by the recycling method of Tietze [40].

BSP concentrations in plasma and bile were determined by spectrophotometry, after appropriate dilution with 0.1 N NaOH. Relative proportions of free and conjugated BSP in bile were assessed by thin layer chromatography, using silica gel as the absorbent and acetone:water:ammonia (81:15:4) as the solvent system [41].

[^{14}C]TC in plasma and bile was measured in a liquid scintillation counter (RackBeta, Pharmacia Wallac Oy), using OptiScint 'Hi Safe' (LKB) as the scintillation cocktail. [^{14}C]TC hepatic content was measured in the supernatant obtained after centrifugation of an homogenate of the major lobe (10% w/v, in saline).

2.7. Statistical analysis

Results were expressed as mean ± SEM. One-way ANOVA, followed by Newman–Keuls test, was performed for multiple comparison among groups. The unpaired Student's *t*-test was used for comparison within two groups. The regression line analysis was done by the least square method, and the significance of the differences between slopes and between *y*-intercepts assayed by covariance analysis using GraphPad Prism software. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of SIL on TLC-induced decrease in BF and BS secretion

Figure 1 shows the time-course changes in BF and total BS output after TLC administration. BF was rapidly

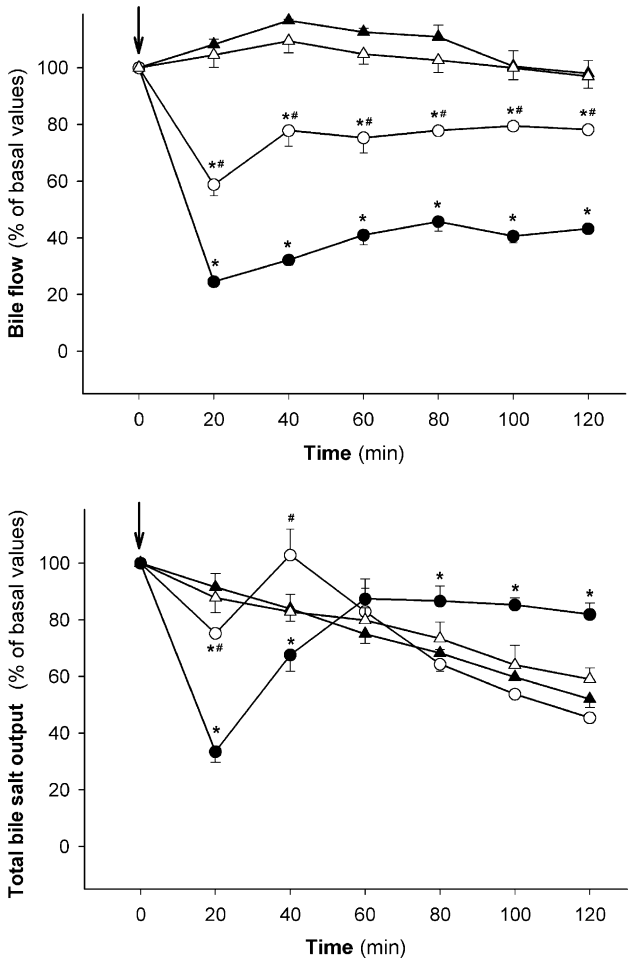

Fig. 1. Time-course changes induced by TLC (3 μmol/100 g body wt, in 10% BSA in saline) in bile flow (upper panel) and total bile salt output (lower panel), referred as percent of basal values, in animals pretreated with SIL (100 mg/kg body wt/day for 5 days, intraperitoneally, ○), or with vehicle (propylene glycol, ●). Control (▲) and SIL (△) animals received only the TLC vehicle (10% BSA in saline). Black arrows indicate either TLC or vehicle administration. Values are expressed as mean ± SEM, for six animals per group. (*) Significantly different from control group ($P < 0.05$). (#) Significantly different from TLC group ($P < 0.05$).

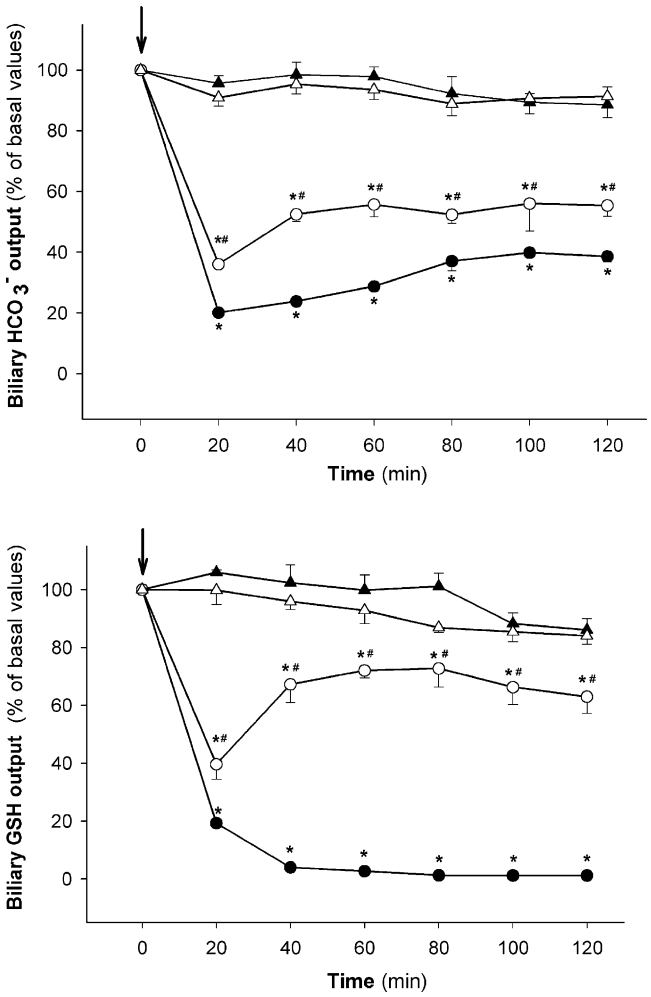

Fig. 2. Time-course changes induced by TLC (3 μmol/100 g body wt, in 10% BSA in saline) in the biliary output of HCO_3^- (upper panel) and GSH (lower panel), referred as percent of basal values, in animals pretreated with SIL (100 mg/kg body wt/day for 5 days, intraperitoneally, ○), or with vehicle (propylene glycol, ●). Control (▲) and SIL (△) animals received only the TLC vehicle (10% BSA in saline). Black arrows indicate either TLC or vehicle administration. Values are expressed as mean ± SEM, for four animals per group. (*) Significantly different from control group ($P < 0.05$). (#) Significantly different from TLC group ($P < 0.05$).

diminished, showing a nadir at 20 min. After that, BF recovered slowly, although it remained significantly lower than controls throughout the experiment. While SIL *per se* did not affect BF, pretreatment of animals with SIL was instrumental in partially preventing the diminution in BF induced by TLC.

BS output was also severely diminished 20 min after TLC administration, but was fully normalized by 60 min after TLC administration, and even overcame the BS output of controls from this latter time onwards; this more likely reflects rapid elimination of the BS retained during the previous secretory failure. The drop in BS output induced by TLC was extensively prevented by SIL; following a slight, short-term decrease in BS output, a transient secretory overshoot occurred, which is also likely due to removal of retained BS.

3.2. Effect of SIL on the decrease of the biliary output of GSH and HCO_3^- induced by TLC

As can be seen in Fig. 2, biliary excretion of HCO_3^- suffered a rapid, long-lasting diminution following TLC administration, although a trend towards recovery was apparent after this initial drop. SIL pretreatment prevented partially the initial decrease in HCO_3^- biliary excretion, which remained higher than TLC group throughout the whole period of time studied. Unlike HCO_3^-, GSH output remained uniformly and extensively impaired during the whole experimental period. SIL significantly prevented the initial decrease in GSH output and, more importantly, induced a rapid recovery, reaching values slightly lower than those from controls.

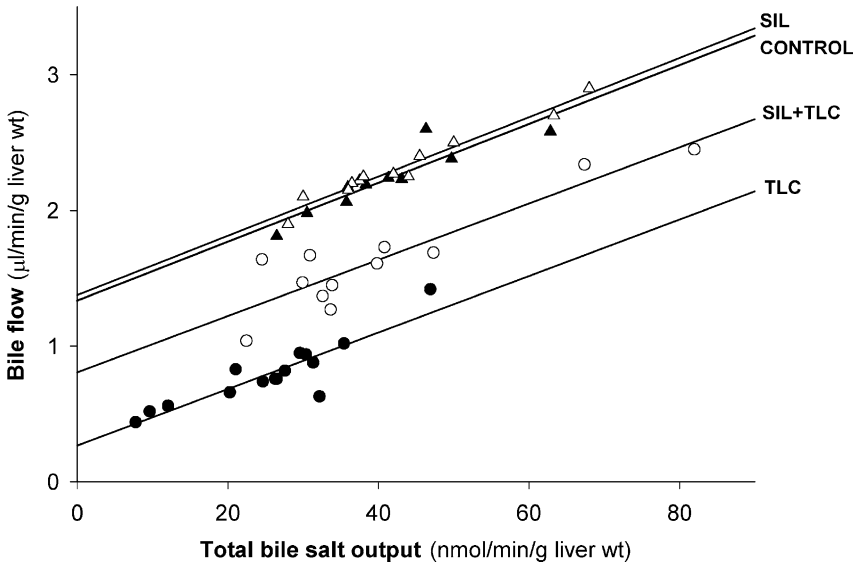

Fig. 3. Relationship between bile flow and bile salt output, as modified by TLC administration (3 μmol/100 g body wt, in 10% BSA in saline), in animals pretreated with SIL (100 mg/kg body wt/day for 5 days, i.p., ○) or with vehicle (propylene glycol, ●). Control (▲) and SIL (△) animals received only the TLC vehicle (10% BSA in saline). The regression lines were obtained by plotting values corresponding to the initial phase of cholestasis, i.e. up to 40 min after TLC injection. Regression analysis yielded the following equations: control: $y = 0.022x + 1.33$; SIL: $y = 0.022x + 1.38$; TLC: $y = 0.021x + 0.27$; SIL + TLC: $y = 0.021x + 0.81$. The correlation coefficient was >0.80 for all groups.

Since excretion of both HCO_3^- and GSH into bile was proposed to be the main driving forces for BSIF formation [42], it was of interest to evaluate this fraction of BF. Therefore, the BSIF estimation was carried out by conventional extrapolation to zero BS output of the regression line between BF and BS output [42]. The regression lines obtained by plotting values corresponding to the initial phase of TLC-induced cholestasis, that is, up to 40 min after injection of the cholestatic, can be seen in Fig. 3. TLC caused an 80% reduction in BSIF, and this impairment was partially prevented by SIL, so that a decrease in BSIF of only 39% was recorded in the rats pretreated with the flavonolignan. In contrast, no change in the choleretic efficiency of BS, as assessed by the slope of these regression lines, was apparent in any of the groups studied, when compared between each other.

3.3. Effect of SIL on the impairment of the hepatic handling of BS and BSP induced by TLC

The influence of the treatments on the status of the hepatic BS and non-BS organic anion transport systems was analyzed by assessing the model-dependent transfer rates derived from the bicompartmental analysis of the plasma decay of BSP and [^{14}C]TC, respectively.

As shown in Table 1, TLC had no effect on the BSP uptake process (as evaluated by r_{12}), in line with previous results by our group [43]. In contrast, TLC significantly increased the liver-plasma reflux process (as assessed by r_{21}, +104%) and impaired markedly the efficiency of the BSP canalicular transfer (r_3, −60%); this may account for the 37% decrease of its cumulative biliary secretion, 60 min after BSP injection.

Table 1
Effect of SIL on the alterations induced by TLC in the hepatic handling of BSP

	Cumulative biliary BSP output (mg/60 min/g liver wt)	Biliary BSP metabolites (% of total BSP)		Intrinsic transfer rates (min^{-1})		
		Free	Conjugated	r_{12}	r_{21}	r_3
Control	1.51 ± 0.04	8.1 ± 1.2	91.9 ± 1.2	0.329 ± 0.017	0.078 ± 0.009	0.121 ± 0.012
SIL	1.51 ± 0.03	6.8 ± 0.8	93.2 ± 0.8	0.278 ± 0.005[a]	0.083 ± 0.003	0.111 ± 0.016
TLC	0.95 ± 0.05[a]	18.6 ± 1.2[a]	81.4 ± 1.2[a]	0.312 ± 0.020	0.159 ± 0.016[a]	0.049 ± 0.006[a]
SIL + TLC	1.54 ± 0.02[b]	8.5 ± 0.6[b]	91.5 ± 0.6[b]	0.279 ± 0.010[a,b]	0.189 ± 0.034[a]	0.112 ± 0.009[b]

Animals belonging to SIL and SIL + TLC groups were pretreated with a daily dose of SIL (100 mg/kg body wt, i.p., for 5 days); control and TLC animals received the SIL vehicle (propylene glycol). Composition of BSP metabolites were determined by thin layer chromatography in bile collected during the period of maximal BSP excretion (i.e. 30–40 min after BSP administration). The transfer rates derived from the compartmental analysis of BSP plasma decay, r_{12}, r_{21}, and r_3, were obtained according to standard equations for an open, bicompartmental model (Richards' model [37]). SIL: silymarin; TLC: taurolithocholate; BSP: sulfobromophthalein; r_{12}: fractional transfer rate for uptake; r_{21}: fractional transfer rate for plasma reflux; r_3: fractional transfer rate for canalicular excretion. Values are expressed as mean ± SEM, for four animals per group.

[a] Different from control group ($P < 0.05$).

[b] Different from TLC group ($P < 0.05$).

Table 2
Effect of SIL on the alterations induced by TLC in the hepatic handling of [^{14}C]TC

	Cumulative biliary [^{14}C]TC output (dpm/20 min/μg liver wt)	[^{14}C]TC retained in liver tissue (dpm/μg liver wt)	Intrinsic transfer rates (min^{-1})		
			r_{12}	r_{21}	r_3
Control	63.1 ± 4.3	1.7 ± 0.5	1.406 ± 0.150	0.090 ± 0.004	0.219 ± 0.021
SIL	66.3 ± 1.9	1.4 ± 0.1	1.249 ± 0.247	0.077 ± 0.029	0.204 ± 0.035
TLC	32.7 ± 0.2[a]	21.8 ± 4.8[a]	1.202 ± 0.144	0.124 ± 0.033	0.069 ± 0.010[a]
SIL + TLC	53.0 ± 5.4[a,b]	11.3 ± 4.5[a,b]	1.299 ± 0.120	0.080 ± 0.005	0.121 ± 0.019[a,b]

Animals belonging to SIL and SIL + TLC groups were pretreated with a daily dose of SIL (100 mg/kg body wt, i.p., for 5 days); control and TLC animals received the vehicle (propylene glycol). The amount of [^{14}C]TC retained in liver tissue was determined in liver homogenates at the end of the experimental period (i.e. 20 min after [^{14}C]TC administration). The transfer rates derived from the compartmental analysis of [^{14}C]TC plasma decay, r_{12}, r_{21}, and r_3, were obtained according to standard equations for an open, bicompartmental model (Richards' model [37]). SIL: silymarin; TLC: taurolithocholate; [^{14}C]TC: [^{14}C]taurocholate; r_{12}: fractional transfer rate for uptake; r_{21}: fractional transfer rate for plasma reflux; r_3: fractional transfer rate for canalicular excretion. Values are expressed as mean ± SEM, for three animals per group.

[a] Different from control group ($P < 0.05$).

[b] Different from TLC group ($P < 0.05$).

Whereas r_{12} was slightly reduced by SIL *per se*; pre-administration of SIL fully prevented the decrease in r_3 induced by the cholestatic. Since canalicular transport represents the rate-limiting step in the overall transport of organic anions from blood to bile, this improvement in r_3 well agreed with normalization of the cumulative biliary excretion of BSP induced by SIL. Contrarily, SIL was unable to prevent the increase in r_{21} induced by TLC. Our finding that SIL was still able to fully prevent the decrease in the cumulative biliary excretion of the BSP (see above) in spite of the high r_{21} value suggests that the increased sinusoidal efflux has a rather marginal, if any, impact on BSP biliary excretion. Table 1 also shows that SIL prevented the increase in the proportion of free BSP appearing in bile induced by TLC.

The model-dependent transfer rates for [^{14}C]TC are shown in Table 2. Whereas r_{12} was not modified, r_3 was decreased by 68% in TLC-treated rats. Although there was a trend towards increased r_{21} in the TLC group, the difference did not achieve statistical significance. These alterations led to a concomitant diminution in the cumulative biliary output of [^{14}C]TC, 20 min after its administration (−48%). The amount of [^{14}C]TC retained in liver tissue was more than 10 times higher in TLC-treated rats than in controls, further suggesting that canalicular transport rather than sinusoidal uptake was the main step impaired. Animals receiving SIL showed a significant improvement in r_3. Since, as for BSP, canalicular transport of BS represents the rate-limiting step in its overall hepatic transport, the partial prevention by SIL of the impairment in r_3 was accompanied by a highly significant recovery in the cumulative excretion of [^{14}C]TC, and in a reduced, but not normalized, accumulation of the radioactive BS in liver tissue at the end of the experimental period (Table 2).

3.4. Effect of SIL on metabolism and biliary excretion of TLC

Figure 4 shows the time-course of biliary excretion of TLC itself and of one of its main phase I metabolites, tauromurideoxycholate (TMDC), after TLC administration; insets depict the cumulative biliary output of these BS at 120 min after administration of the cholestatic. TLC, which is not detected in normal bile, is excreted acutely after a TLC pulse in control rats. SIL pretreatment was instrumental in markedly accelerating biliary excretion of TLC, so that a noticeable secretory peak was apparent, as soon as 20 min after its administration (Fig. 4, upper panel); this led to an increase in the cumulative TLC biliary output of 104% throughout the experimental period (Fig. 4, upper panel, inset). Biliary excretion of TMDC, which was also not detectable in basal conditions, reached a maximum at 40–60 min after TLC administration in TLC-treated rats, and SIL pretreatment was also able to accelerate its biliary output, particularly during the first collection periods (Fig. 4, lower panel); this led to a significant increment in the cumulative biliary output of TMDC of 70% (Fig. 4, lower panel, inset).

Cumulative biliary output of taurine conjugates of CDC, β-MC and hyodeoxycholate, resulting from TLC phase I metabolism [32,33], as discriminated from changes in their excretions from endogenous sources using the non-TLC metabolite, cholate, as an internal standard [32], tended to be higher in SIL + TLC group than in rats only treated with TLC, but these results did not reach statistical significance (data not shown).

4. Discussion

The main finding of the present work is that the hepatoprotector SIL significantly prevents the acute cholestasis induced by a single, i.v. dose of TLC. SIL prevented TLC-induced decrease in BF by partially counteracting the impairment in both BSDF and BSIF formation; the latter effect occurred by improving the biliary excretion of HCO_3^- and GSH, the two main determinants of this fraction of BF.

Our pharmacokinetic studies revealed that TLC affected the hepatic handling of BS and non-BS organic anions,

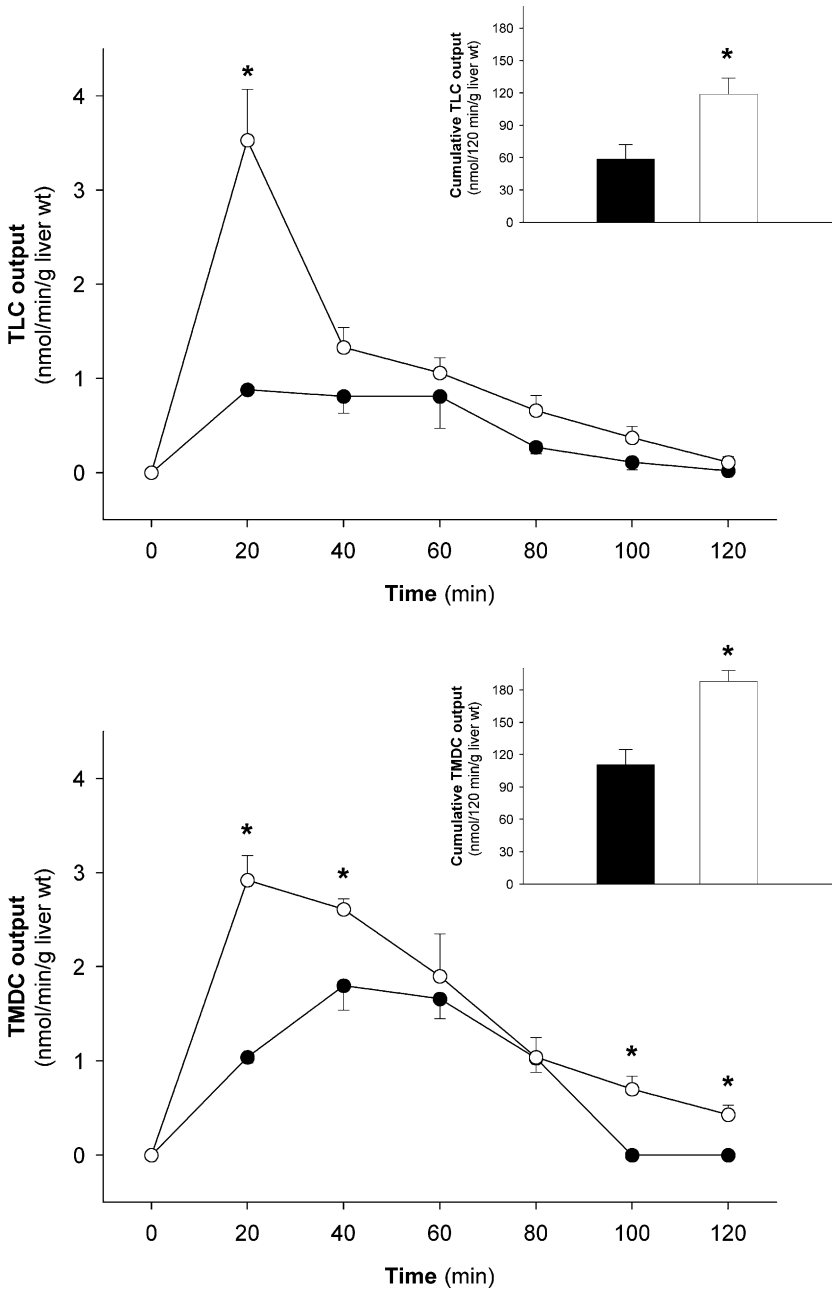

Fig. 4. Time-course changes in the biliary output of TLC (upper panel) and of its hydroxylated metabolite TMDC (lower panel), following TLC administration (3 μmol/100 g body wt, in 10% BSA in saline), in animals pretreated with SIL (100 mg/kg body wt/day for 5 days, i.p., ○) or in controls receiving only the SIL vehicle (propylene glycol, ●). Insets show the cumulative biliary output of these bile salts at 120 min after TLC administration in animals pretreated with SIL (open bars) or in controls receiving only the SIL vehicle (solid bars). (*) Significantly different from TLC group ($P < 0.05$).

mainly by impairing the function of the transport systems at the canalicular level. This was suggested by the significant alteration in the intrinsic transfer constants for canalicular excretion (r_3) of the model non-BS organic anion, BSP and the radioactive BS, [^{14}C]TC (see Tables 1 and 2, respectively); a similar functional impairment in the canalicular transfer of BSP [43] and of another Mrp2 substrate, dinitrophenyl-*S*-glutathione [14], had been previously shown to occur in TLC-induced cholestasis. These alterations can account for the impaired transport of bile-forming solutes thought to be normally excreted into the canaliculus by these transport systems; i.e endogenous BS and GSH, which are transported across the canalicular membrane *via* Bsep [44] and Mrp2 [13].

The mechanism by which TLC impairs the canalicular transport can be multifactorial. TLC selectively decreases the fluidity of the canalicular membrane, which has been shown to alter the canalicular transport activity for BS and, probably, other solutes [45]. It has been also described that TLC affects the normal, canalicular localization of transport proteins, including Mrp2 [14] and Bsep [15], which are endocyted in intracellular, vesicular-like structures.

Consequently, loss of transporters from the canalicular membrane could be responsible, at least in part, for the decrease in the constant of canalicular transport, r_3, observed here under TLC treatment. This effect may not be restricted to these two transport systems, but may include other transport systems thought to be involved in bile formation, like the Cl^-/HCO_3^- exchange system, AE2, which is responsible for HCO_3^- excretion into bile [46]. Indeed, TLC was shown to induce a non-selective relocalization of canalicular membrane-bound proteins to the sinusoidal pole in cultured hepatocytes, as revealed by ultrastructural studies [47].

The pharmacokinetic studies carried out here also revealed conclusively that SIL pretreatment significantly ameliorated the function of the transport systems at the canalicular level as impaired by TLC; this was suggested by the significant improvement of the intrinsic transfer constants for canalicular excretion for both BSP and [^{14}C]TC (see Tables 1 and 2, respectively).

Our finding that SIL, when administered chronically *in vivo*, improved metabolic detoxification of TLC (see Fig. 4) provides insight into its protective mechanism. Several lines of evidence have indicated that, in addition to the elimination of TLC itself into bile, phase I metabolism which renders TLC non-cholestatic, would play an important role in the recovery from cholestasis [34]. Indeed, *in vivo* and *in vitro* studies have shown that 6β-hydroxylation is the predominant metabolism involved in TLC detoxification in rats and mice, leading to the formation of non-cholestatic metabolites, like tauro-conjugates of MDC and β-MC [32,33]. Our finding that SIL pretreatment accelerated both excretion of TLC itself and conversion of the cholestatic to TMDC during the initial phase of cholestasis, and not during the subsequent collection periods, suggests that SIL protective effect occurs by preventing TLC from exerting its cholestatic effect, by reducing its hepatocellular transit time and/or its intracellular level, rather than by accelerating the recovery from injury. Our results that SIL enhanced TLC 6β-hydroxylation are closely in line with our previous finding that the same *in vivo* administration protocol of SIL, under normal conditions, selectively increases the formation of α-MC and β-MC, two BS derived from 6β-hydroxylation of CDC [26]. Although a similar final conversion to MCs is also expected to occur to the exogenously administered TLC (*via* 6β-hydroxylation and subsequent 7β-hydroxylation), our experimental setup with interrupted enterohepatic circulation, which avoids re-exposure to hydroxylating enzymes, may have minimized further 7β-hydroxylation of MDC to β-MC.

Whereas the relevance of the TLC detoxifying pathway involving 6β-hydroxylation may be substantial in rats and mice, its extrapolation to humans is doubtful, as 6β-hydroxylated BS are virtually absent in this species [48]. Rather, humans hydroxylate LC preferentially in 6α position *via* the cytochrome P450 isoenzyme, CYP3A4, [49,50], although some lithocholate-6β-hydroxylating activity by human microsomes is also apparent [50]. Although SIL was found to inhibit rather than to enhance the activity of CYP3A4 when administered both to cultured human hepatocytes [51] and to rats *in vivo* [25], its effect on others putative 6β-hydroxylating systems remains to be ascertained.

In addition to improving TLC hydroxylation, SIL enhanced biliary excretion of non-metabolized TLC, a finding in line with our pharmacokinetic results showing that the flavonolignan preserved the activity of the BS transport system at the canalicular level (see Table 2). Although the mechanism/s by which SIL exerts this protective effect cannot be addressed from our results, a likely possibility is that SIL improves the canalicular transport of exogenous and endogenous compounds by preserving their transport proteins at this level. In line with this possibility, we have recently described that silibinin, the major, active component of SIL, when administered acutely to isolated rat hepatocyte couplets, prevents endocytic internalization of Bsep induced by TLC [52]. Whether a similar effect also occurs following chronic administration of SIL *in vivo* remains to be investigated. If so, the protective effects of SIL may occur by two mechanisms acting in concert, namely: enhanced TLC detoxification and prevention of TLC-induced canalicular carrier internalization. In line with this view, administration of SIL *in vivo* increases the size of the pool of MCs and ursodeoxycholate [26], which were shown to have beneficial effects in TLC-induced cholestasis, [30,31], including an amelioration in Bsep function [31]. On the contrary, the possibility that SIL exerts its beneficial effect by preventing TLC-induced increase in cholesterol content of the canalicular membrane and its further rigidization [11] appears unlikely, as we have previously shown that SIL, when administered at the same dosage as that used here, decreased rather than increased plasma membrane fluidity, and was unable to prevent ethynylestradiol-induced plasma membrane rigidization [25].

In summary, SIL exhibits a beneficial effect against TLC-induced impairment of both BSDF and BSIF formation by preventing the alteration in the biliary excretion of endogenous BS, GSH, and HCO_3^-, the three major determinants of these fractions of the BF. SIL exerts its beneficial effect, at least in part, by enhancing TLC excretion itself, an effect closely linked to its ability to preserve the activity of the BS transport system at the canalicular level, and by accelerating TLC conversion to hydroxylated, non-cholestatic metabolites.

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