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Effect of Silymarin on Biliary Bile Salt Secretion in the Rat

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ABSTRACT. The effect of the hepatoprotector silymarin on bile secretion, with particular regard to bile salt secretion, was studied in Wistar rats. Silymarin (25, 50, 100, and 150 mg/kg/day, i.p., for 5 days) induced a dose-dependent increase in bile flow and bile salt secretion, the maximal effect being reached at a dose of 100 mg/kg/day (+17 and +49%, for bile flow and bile salt output, respectively; $P < 0.05$). Assessment of bile salt composition in bile revealed that stimulation of the bile salt secretion was accounted for mainly by an increase in the biliary secretion of β-muricholate and, to a lesser extent, of α-muricholate, chenodeoxycholate, ursodeoxycholate, and deoxycholate. The maximum secretory rate (T_m) of bile salts, as assessed by infusing the non-hepatotoxic bile salt tauroursodeoxycholate i.v. at stepwise-increasing rates, was not influenced by silymarin. The flavonolignan also increased the endogenous bile salt pool size (+53%, $P < 0.05$) and biliary bile acid excretion after bile acid pool depletion (+54%, $P < 0.05$), a measure of *de novo* bile salt synthesis. These results suggest that silymarin increases the biliary excretion and the endogenous pool of bile salts by stimulating the synthesis, among others, of hepatoprotective bile salts, such as β-muricholate and ursodeoxycholate. BIOCHEM PHARMACOL **59**;8:1015–1022, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. silymarin; hepatoprotection; bile secretion; bile salt output; ursodeoxycholate; muricholate

SIL† is a purified extract from the milk thistle *Silybum marianum* (L.) Gaertn; it is composed of a mixture of three isomeric flavonolignans: silibinin, silidianin, and silicristin, the former being the most active component [1]. 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 (recently reviewed by Flora *et al.* [1]). In addition, SIL has been shown to protect experimental animals from various hepatotoxicants, including carbon tetrachloride [2, 3], acetaminophen [4], phalloidin [5], D-galactosamine [3], and allyl alcohol [3], among others.

The mechanisms by which SIL exerts its hepatoprotective action are under intensive investigation, and appear to be multifactorial in origin. SIL stabilizes membranes, making cells more resistant to osmotic lysis and to the action of detergents [6]. This flavonolignan prevents lipid peroxide formation in liver cells [7], due, at least in part, to its properties as a free radical scavenger [8]. In addition, the active component of SIL, silibinin, has been shown to inhibit the function of Kupffer cells, which are well-recognized sources of fibrogenic mediators [9]. This would help to explain the antifibrotic properties of SIL in biliary cirrhosis secondary to biliary obstruction in rats [10] and, perhaps, the improvement in parenchymal alterations and in portal inflammation observed in patients with chronic hepatitis [11]. Finally, SIL was shown to be a potent protein synthesis inducer via stimulation of RNA synthesis [12]; this could account in large part for its beneficial effect on liver regeneration [13] and its capability to increase activity of the mixed-function oxidation system [14].

Bile salts are chief constituents of bile. They play a crucial role both in bile formation and, once delivered to the intestine, in lipid absorption by the intestinal tract [15]. Hepatic accumulation of bile salts due to secretory alterations, either by mechanical or functional causes, is thought to have a crucial role in the initiation and/or perpetuation of liver injury [16]. Indeed, bile salts are amphipathic compounds that, due to their detergent properties, can disrupt membrane structure and alter permeability to hepatocellular constituents [17]. Furthermore, bile salts are able to uncouple oxidative phosphorylation, thus inducing oxidative stress and other cytotoxic damages [18]. However, it has also become increasingly apparent that, under such conditions, beneficial changes in the pattern of bile salt metabolism may occur either spontaneously, as an adaptive mechanism in liver pathologies, or due to iatrogenic causes, secondary to therapy.

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† *Abbreviations*: SIL, silymarin; MC, muricholate; CDC, chenodeoxycholate; UDC, ursodeoxycholate; TUDC, tauroursodeoxycholate; DC, deoxycholate; HDC, hyodeoxycholate; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; and ALP, alkaline phosphatase.

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Adaptive changes in the biliary patterns of bile acids, not only reducing bile salt toxicity but, in some cases, protecting against bile salt toxicity, have been observed in cholestatic diseases, such as those caused by estrogens [19], α-naphthylisothiocyanate [20, 21], methyltestosterone [21], and bile-duct obstruction [16, 22]. In the rat, these changes involve formation of bile salts known to have hepatoprotective effects against bile salt toxicity, such as MC and UDC [23]. In humans with cholestasis, sulfation and glucuronidation, two trace metabolic pathways for endogenous natural bile acids in normal conditions, become prominent, thus contributing to their rapid urinary elimination [24].

Beneficial changes in bile salt composition can also be produced therapeutically. For example, administration of UDC, a tool of choice for the treatment of a large number of cholestatic liver diseases [25], induces an enrichment in the systemic and hepatic content of this non-toxic, hepatoprotective bile salt, with displacement of other potentially toxic endogenous bile salts [25]. In addition, UDC, as well as rifampicin, a therapeutic agent, ameliorates itching in cholestatic patients, and stimulates hepatic bile acid 6α-hydroxylation and, consequently, urinary excretion of "unusual" trihydroxy bile acids, such as α- and β-hyocholate, and ursocholate [26, 27]; with the addition of a 6α-hydroxy group, hydrophobic and potentially cytotoxic bile acids are converted into their hydrophilic derivatives, which can be glucuronidated and excreted into the urine [28].

The recent reemergence of SIL for the treatment of liver diseases and the recognition that beneficial effects of therapeutic agents may be mediated, at least in part, by beneficial changes in bile acid excretion and/or bile acid pool composition have prompted our examination of the effect of SIL on these parameters. Our results provide evidence that SIL induces changes in the magnitude and composition of the bile acid pool that could be relevant to its hepatoprotective properties.

MATERIALS AND METHODS

Chemicals

SIL and 3α-hydroxysteroid dehydrogenase were obtained from the Sigma Chemical Co. Sodium TUDC was a gift from Prodotti Chimici e Alimentari S.p.A. This bile salt was more than 98% pure when examined by HPLC. All the other reagents were of the highest analytical grade available from commercial sources.

Animals and Treatments

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 lib.* and were housed in a temperature- (21–23°) and humidity- (45–50%) controlled room under a constant 12-hr light, 12-hr dark cycle.

Animals were divided randomly into five experimental groups. Four of the groups received daily SIL doses of 25, 50, 100, or 150 mg/kg/day, i.p., for 5 consecutive days; the fifth group (control) received only the vehicle (propylene glycol).

Surgical procedures were performed on day 6. Bile collection was started between 9:00 a.m. and 11:00 a.m. to minimize the influence of circadian variations. Animals were anesthetized with a single dose of sodium pentobarbital (50 mg/kg body weight, i.p.), and were maintained under this condition throughout the experiment. After catheterization of the femoral artery and femoral vein using PC-50 polyethylene tubing (Intramedic, Clay Adams), a middle abdominal incision was made, and the common bile duct was also cannulated (PE-50, Intramedic, Clay Adams). Tracheal cannulation was performed systematically to remove bronchial secretions induced by the anesthetic. Body temperature was maintained at 37.5 to 38.0° with a warming lamp to prevent hypothermic alterations of bile flow. At the end of each experiment, animals were killed by exsanguination, and the liver was removed and weighed.

Experimental Procedure

Bile fistula rats were randomized to one of the following groups:

BASAL RATS. These animals were employed in dose–response studies. For this purpose, spontaneously secreted bile was collected for 30 min in pre-weighed vials. Bile was previously allowed to drain for 30 min to ensure a stable bile flow.

The remaining experiments were performed using a dose of silymarin of 100 mg/kg/day, since an apparent maximal effect was reached at this dose (see below).

TUDC-INFUSED RATS. These animals were used to estimate the maximal capability of the liver to transport bile salts into bile (T_m). TUDC was selected for this purpose due to its extremely low toxicity, in contrast to other naturally occurring bile salts, whose apparent maximum transport is determined largely by the cytotoxic nature of the bile salt rather than by the saturation of its canalicular carrier [29]. The T_m of TUDC was assessed by infusing i.v. this bile salt, dissolved in 2% BSA in saline, at stepwise-increasing rates (2.0, 2.5, 5.0, 6.5, 12.0, and 16.0 μmol/min/100 g body weight). Each infusion rate was maintained for 30 min, and bile samples were collected every 10 min for 180 min. T_m was calculated as the mean of the three highest consecutive 10-min secretory rates recorded over the whole infusion period [29].

BILE SALT-DEPLETED RATS. These animals were employed to estimate bile salt pool size and *de novo* synthesis rate. The chronic bile fistula model provides a means for rapidly depleting the body of bile salts by eliminating the primary source of these compounds, i.e. the enterohepatic circulation [30]. The bile salts excreted during the first 8 hr of bile drainage, when a complete bile salt pool washout was

TABLE 1. Effect of SIL treatment on body and liver weight

	Initial body wt (g)	Final body wt (g)	Change of body wt (%)	Liver wt (g)	Final liver/body wt ratio (%)
Control	330 ± 6	332 ± 6	+0.61 ± 0.04	11.0 ± 1.1	3.3 ± 0.2
SIL (mg/kg body wt)					
25	326 ± 7	320 ± 7	−1.84 ± 0.08	10.9 ± 1.5	3.4 ± 0.2
50	332 ± 9	320 ± 8*	−3.61 ± 0.12	11.1 ± 0.8	3.5 ± 0.3
100	329 ± 8	303 ± 7*†	−7.90 ± 0.25	11.0 ± 1.0	3.7 ± 0.2†
150	325 ± 8	294 ± 8*†	−9.54 ± 0.29	10.9 ± 1.2	3.7 ± 0.2†

Animals received a daily i.p. dose of SIL (25, 50, 100, and 150 mg/kg body wt) for 5 days, or only the vehicle (propylene glycol) in controls. Results are expressed as means ± SEM for 4–6 experiments.

*Significantly different from initial values ($P < 0.05$).

†Significantly different from the control group ($P < 0.05$).

reached, represent the bile salt pool size; bile salt output after this period of time estimates the bile salt synthesis rate [31]. For this purpose, animals were put in restraining cages, and losses of water, electrolytes, and proteins were compensated for by i.v. infusion of a Krebs–Henseleit–bicarbonate solution (pH = 7.4) containing 3 mg/mL of glucose and 2 mg/mL of BSA; then, they were subjected to continuous biliary drainage for 12 hr, overnight. No further decrease in bile salt output was observed after 8 hr, indicating that a complete bile salt pool washout was reached at this time, as reported by others [30].

Analytical Procedures

The volume of bile was determined by weight, assuming a density of 1.0 g/mL. The biliary excretion rate was calculated as the product of bile flow and solute concentration values. Distortion of excretory curves produced by the biliary tree dead space was corrected as previously described [32].

Total bile salts in bile were measured by the 3α-hydroxysteroid dehydrogenase procedure [33]. Biliary bile salt composition was assessed by HPLC (Waters) as reported previously [34]. Individual bile salts were identified by using appropriate standards.

Serum activities of ASAT (EC 2.6.1.1) and ALAT (EC 2.6.1.2) were assessed spectrophotometrically by measuring NADH consumption at 340 nm. ALP (EC 3.1.3.1) was assessed using *p*-nitrophenyl phosphate as a substrate. In all cases, commercial kits were used (Wiener Lab.).

Statistical Analysis

Results were expressed as means ± SEM. Means of two groups were compared with Student's *t*-test after testing the equality of variances with an *F*-test. Multiple means were compared by using one-way ANOVA followed by Student's *t*-test for pairwise comparisons. *P* values lower than 0.05 were judged to be significant.

RESULTS

SIL had little or no impact on liver integrity, as suggested by the lack of effect of SIL on the serum biochemical markers of hepatocellular injury studied, i.e. ASAT, ALAT, and ALP, at any of the doses tested (data not shown). However, a progressive, dose-dependent decrease in total body weight was recorded during the treatment (Table 1). Hence, although no significant difference in liver weight was found between SIL-treated and control rats at any dose level, the final liver-to-body weight ratio increased also in a dose-dependent manner.

As shown in Fig. 1, treatment of the animals with increasing doses of SIL induced a progressive increase in both basal bile flow and total bile salt output. An apparent maximal effect was reached at the dose of 100 mg/kg, which amounted to an increment of 17 and 49%, for bile flow and bile salt output, respectively. A careful analysis of the basal biliary output of individual bile salts (Table 2) revealed that the increase in total bile salt output induced by SIL (100 mg/kg) was accounted for mainly by the rise in the primary bile salt β-MC, although higher outputs of α-MC, UDC, and CDC, as well as of the secondary bile salt DC, were also recorded. A trend towards higher HDC excretion was also apparent, but the difference did not achieve statistical significance, compared with controls. A further analysis of the relative increments of these individual bile salts in bile revealed a preferential percent increase for α-MC, β-MC, HDC, UDC, and DC (+87, +71, +85, +72, and +108%, respectively), and a lower but significant increment for CDC (+35%). The selective increment of these bile salts led to a relative decrease in the proportion of cholate in bile (from 33.2 ± 3.2 to 22.8 ± 1.4%, $P < 0.01$), although its absolute biliary output was not affected by SIL. The treatment also did not modify the ratio of glycine- to taurine-conjugated bile salts.

The size of the endogenous bile salt pool was increased by 53% by SIL administration (Table 3). This increment was accounted for mainly by an increased amount of α-MC, β-MC, UDC, HDC, and DC (+101, +76, +98, +79, and +92%, respectively) and, to a lesser extent, CDC (+36%).

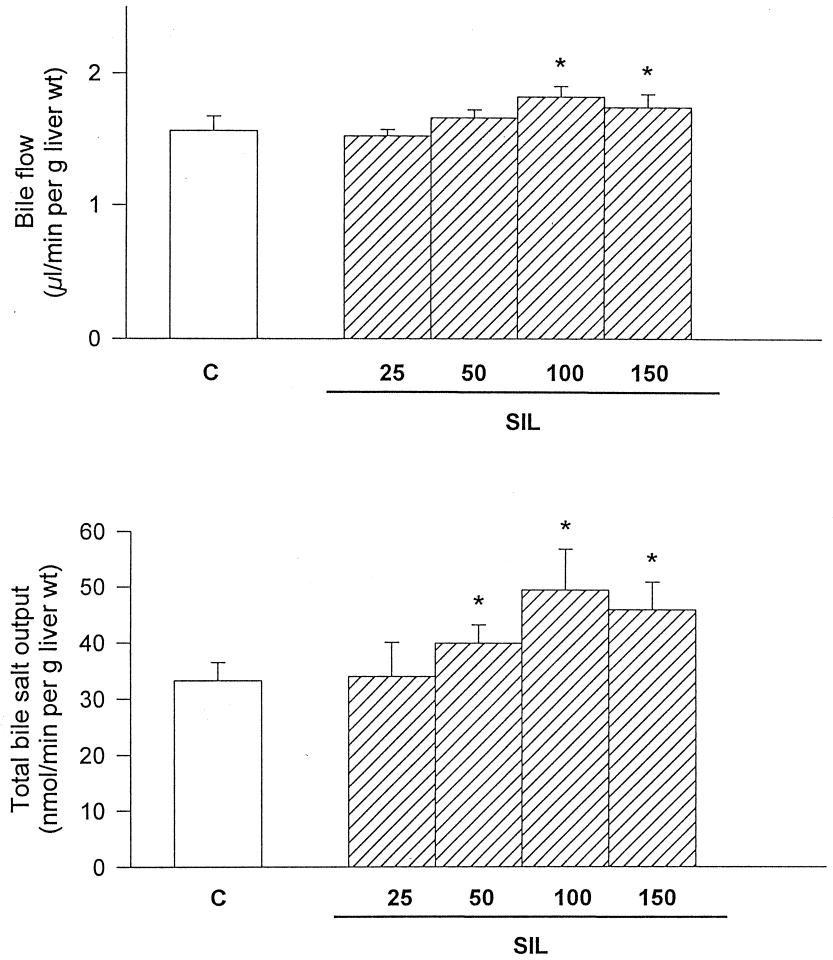

FIG. 1. Effect of SIL on bile flow (upper panel) and total bile salt output (lower panel). These parameters were assessed following the administration of SIL (25, 50, 100, or 150 mg/kg body weight, in propylene glycol, i.p.), or only the vehicle in controls (C). Values were expressed as means ± SEM of 6 animals in control and the 100 mg/kg body weight SIL group, and of 4 animals for the remaining groups. Key: (*) significantly different from the control group ($P < 0.05$).

On the other hand, the content of cholate and the ratio of glycine-conjugated to taurine-conjugated bile salts in the bile salt pool was unaffected by SIL. Bile salt output following an 8-hr depletion of the bile salt pool, a parameter that reflects the *de novo* bile salt synthesis rate [31], was increased by 100 mg/kg body weight of SIL (6.1 ± 0.7 and 9.4 ± 0.6 nmol/min/g liver for controls and SIL-treated rats, respectively, $P < 0.05$).

As can be seen in Fig. 2, the T_m of the model bile salt TUDC was not influenced by SIL pretreatment (770 ± 64 and 784 ± 16 nmol/min/g liver for controls and SIL-treated rats, respectively). When the relationship between bile flow and bile salt output obtained during TUDC infusion was examined (Fig. 2), no significant difference was observed between SIL-treated and control rats in either the y-intercept or the slope of the regression line, which assesses

TABLE 2. Effect of SIL on basal bile salt output

	Bile flow (μL/min per g liver wt)	Total bile salt output (nmol/min per g liver wt)	Individual bile salt output (nmol/min per g liver wt)							
			α-MC	β-MC	UDC	HDC	C	CDC	DC	G/T ratio
Control	1.56 ± 0.11	33.3 ± 3.2	2.3 ± 0.3	12.6 ± 1.7	1.8 ± 0.3	2.0 ± 0.3	11.1 ± 1.5	2.3 ± 0.3	1.2 ± 0.2	0.10 ± 0.03
SIL	1.82 ± 0.10*	49.5 ± 7.4*	4.3 ± 0.4*	21.6 ± 3.8*	3.1 ± 0.4*	2.7 ± 0.5	11.3 ± 2.2	3.1 ± 0.3*	2.5 ± 0.6*	0.12 ± 0.03

Bile flow and bile salt output were measured in basal bile of none-bile-salt-depleted animals treated with a daily i.p. dose of SIL (100 mg/kg body wt) for 5 days, or in control animals receiving only the vehicle (propylene glycol). α-MC: α-muricholate; β-MC: β-muricholate; HDC: hyodeoxycholate; UDC: ursodeoxycholate; C: cholate; CDC: chenodeoxycholate; DC: deoxycholate; G/T ratio: ratio of glycine- to taurine-conjugated bile salts. Results are expressed as means ± SEM for 6 experiments.

*Significantly different from the control group ($P < 0.05$).

TABLE 3. Effect of SIL on endogenous bile salt pool size and composition

	Bile salt pool size (μmol/kg body wt)	Individual bile salt amount in the bile salt pool (μmol/kg body wt)							
		α-MC	β-MC	UDC	HDC	C	CDC	DC	G/T ratio
Control	290 ± 30	25.1 ± 2.8	114.9 ± 8.4	10.5 ± 0.4	14.1 ± 2.4	95.0 ± 9.5	24.2 ± 3.4	6.4 ± 0.9	0.12 ± 0.03
SIL	443 ± 50*	50.5 ± 5.2*	201.8 ± 10.5*	20.8 ± 1.2*	25.2 ± 1.7*	99.7 ± 6.9	33.0 ± 5.6*	12.3 ± 2.5*	0.13 ± 0.04

Bile salt pool size and composition were measured in bile of chronically drained animals pretreated with a daily i.p. dose of SIL (100 mg/kg body wt) for 5 days, or in control animals receiving only the vehicle (propylene glycol). α-MC; α-muricholate; β-MC: β-muricholate; HDC: hyodeoxycholate; UDC: ursodeoxycholate; C: cholate; CDC: chenodeoxycholate; DC: deoxycholate; G/T ratio: ratio of glycine- to taurine-conjugated bile salts. Results are expressed as means ± SEM for 6 experiments.

*Significantly different from the control group ($P < 0.05$).

the bile salt-independent fraction of bile flow and the choleretic efficiency of the secreted bile salts, respectively [35].

DISCUSSION

In this study, we described a number of effects induced by SIL on biliary secretion that may be relevant to the understanding of the mechanisms involved in its hepatoprotective effects.

A 5-day treatment with SIL induced a dose-dependent stimulatory effect on bile flow and bile salt output (see Fig. 1). Conversely, no change in the bile salt-independent fraction of the bile flow, as measured by the y-intercept of the regression line between bile flow and the bile salt output stimulated by TUDC (see Fig. 2), was recorded. SIL flavonolignans have been reported to be partially glucuronidated and further excreted into bile [36]. However, it is possible that the dose used in this study was too low to induce significant osmotic choleresis, as biliary excretion of its components was reported to be only 0.001% of the dose for silibinin [36]. Therefore, the choleretic effect of SIL was fully accounted for by an increment in bile salt output, which induced a dose-dependent increase in the bile-salt dependent fraction of the bile flow. Since the T_m for the model bile salt TUDC was not affected by SIL (see Fig. 2), its stimulatory effect on bile salt secretion appears not to be due to an increased capability of the canalicular transport system for bile salts, the rate-limiting step in the handling of bile salts by the liver.

Our findings that SIL increased both bile flow and bile salt output seem to be in contradiction to a previous study, which showed that silibinin, a component of SIL that is thought to be the more active one, had no effect on any of these parameters when administered at a similar dose to that used here for SIL, i.e. 100 mg/kg/day [37]. It would seem likely, therefore, that the remaining components of SIL are responsible for these differential effects, somehow modulating the silibinin effect either directly or indirectly.

In an attempt to further characterize the mechanisms involved in SIL-induced stimulation of bile salt output, we evaluated the influence of the flavonolignan on the size of the bile salt pool. Bearing in mind that bile salt output is the product between bile salt pool size and the fraction of times that this pool recirculates per unit time [38], an increased bile salt pool should lead to an increased bile salt output. In line with this possibility, the size of the bile salt pool was increased significantly by SIL treatment (see Table 3). Furthermore, the percent increment in the bile salt pool (+53%) was in reasonably good agreement with that of the bile salt output (+49%), suggesting that a causal link does exist. Interestingly, the change recorded in the output of each individual bile salt following SIL administration closely reflected their respective increment in the bile salt pool.

The size of the endogenous bile salt pool represents a balance among bile salt synthesis, biliary secretion, and intestinal absorption. Intestinal transport of taurine-conjugated bile salts, by far the major bile salts in the rat, is mediated entirely by the ileal Na^+-dependent transport system [39]. Therefore, putative changes in the activity of this single transporter are unlikely to selectively modify the proportions of only certain bile salts, as SIL did (see Table 3). Furthermore, this carrier is known to transport cholyl conjugates more efficiently than dihydroxy conjugates [39]; if SIL had stimulated this transport system, an increase of cholate in the bile salt pool content would have been expected to occur. Bearing in mind that, in turn, biliary bile salt output was increased rather than decreased by SIL, the increase in bile salt pool size was more likely due to increased bile salt synthesis. This was supported further by the finding that SIL increased bile salt output after bile salt pool depletion, a parameter that reflects the *de novo* bile salt synthesis rate [31].

When individual increments of the amount of each bile salt in the bile salt pool are considered (see Table 3), it becomes apparent that an increase of either CDC itself or other bile salts derived from its further hepatic or intestinal conversion did occur, suggesting that the pathways involved in CDC synthesis were preferentially stimulated by SIL. Indeed, ~93% of the increment of the biliary bile salt pool in the SIL-treated group was accounted for by CDC together with its metabolically derived compounds, i.e. α-MC (formed by hepatic CDC 6β-hydroxylation), β-MC (formed by further α-MC 7-epimerization), UDC (mainly formed by combined intestinal CDC 7α-dehydrogenation and further hepatic reduction of the 7-oxo derivative), and HDC (the secondary bile salt derived from intestinal

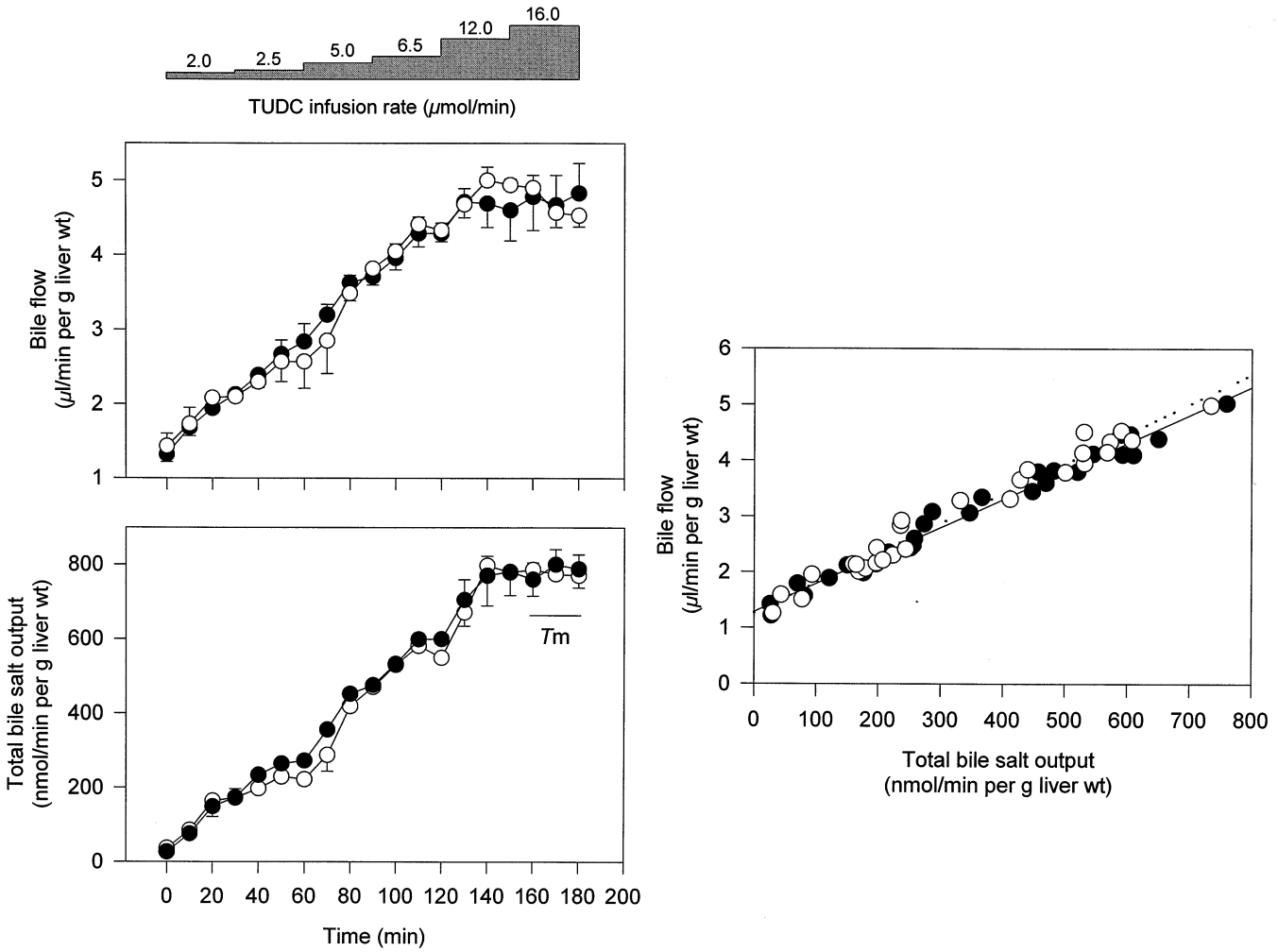

FIG. 2. Sequential changes in bile flow (left, upper panel), total bile salt output (left, lower panel), and the relationship between these two parameters (right) in rats treated with SIL (100 mg/kg body weight) for 5 days (○), or in control rats receiving only the vehicle (●). Animals were infused i.v. with TUDC at stepwise-increasing rates (2.0, 2.5, 5.0, 6.5, 12.0, and 16.0 μmol/min). The T_m of TUDC, calculated as the mean of the three highest consecutive secretory rates, was 770 ± 64 and 784 ± 16 nmol/min/g liver for controls and SIL-treated rats, respectively. The regression lines obtained when bile flow was plotted against total bile salt output were y = 1.284 + 0.005x (r^2 = 0.979) and y = 1.266 + 0.005x (r^2 = 0.970) for controls and SIL-treated rats, respectively. All values are means ± SEM of 4 experiments.

conversion of MCs) [40]. As a result, the CDC group/cholate group ratio increased in the bile salt pool from 1.96 to 2.96 following SIL administration, suggesting a preferential stimulation of the CDC synthetic pathway over that of cholate. Although the mechanisms by which these changes were brought about are speculative at present, it is worth noting that SIL is a protein synthesis inducer [12]; this could account in large part for its stimulatory effect on bile salt synthesis. Interestingly, phenobarbital, another protein synthesis inducer that, like SIL, is known to increase the liver microsomal hydroxylating activity of drugs and other substrates including steroids, was shown to expand the bile salt pool by selectively increasing bile salts belonging to the CDC group [41]. A key point in the regulation of bile salt synthesis is the microsomal enzyme cholesterol 7α-hydroxylase, the initial, rate-limiting enzyme controlling the overall (cholate plus CDC) bile salt synthesis [40, 42]. If SIL stimulates bile salt synthesis at this level, however, simultaneous inhibition of 12α-hydroxylase, the enzyme that catalyzes the conversion of the common cholate and CDC precursor 7α-hydroxy-4-cholesten-3-one to 7α,12α-dihydroxy-4-cholesten-3-one, thus leading to irreversible cholate formation, should occur. Inhibition of 12α-hydroxylase activity has been reported to occur following either CDC or thyroid hormone administration, two manipulations that, like SIL administration, lead to a marked increment of the CDC group/cholate group ratio [42]. However, activation of the alternative biosynthetic pathway of CDC biosynthesis, involving oxidation of the side chain before changes in the steroid nucleus occur, cannot be excluded, although the quantitative importance of this pathway to overall bile salt synthesis is thought to be minor [42]. Whatever may be the pathway involved, the finding that SIL improved bile salt excretion by expanding the

size of the bile salt pool may have considerable biological relevance to the situation in human beings, since the sequence of CDC formation via both the classical and the alternative pathways in humans is similar qualitatively to that in the rat [40].

Another interesting observation was that, whereas SIL treatment led to only a 36% increment of the amount of CDC in the bile salt pool, bile salts derived from its further conversion to more hydrophilic compounds via 6β-hydroxylation, i.e. α-MC and β-MC, were increased to a far greater extent (+101 and +76%, respectively). This suggests that 6β-hydroxylating activity is also stimulated by the flavonolignan. Similarly, UDC, a bile salt that can be formed either in the liver from cholesterol or in the intestine, by bacterial 7α-hydroxysteroid dehydrogenation of CDC and further reduction of the 7-oxo acid in the liver [24], was increased to a far greater extent than CDC, and the same holds true for HDC, the secondary bile salt derived from intestinal MC conversion (+98 and +79%, respectively). Interestingly, α-MC, β-MC, and UDC [23], together with HDC [43], share hepatoprotective properties against the hepatocellular damage induced by more hydrophobic bile salts, as well as against a variety of drug-induced toxicological and cholestatic insults [44]. The finding that SIL was able to increase the bile salt pool mainly by increasing these hepatoprotective bile salts opens the possibility that the flavonolignan may induce hepatoprotection, at least in part, via these bile salts. Whereas the relevance of this beneficial mechanism may be substantial in rats and mice, which metabolize CDC to α-MC and β-MC, its extrapolation to human beings is doubtful, as humans lack 6β-hydroxylase activity [40]. Therefore, caution should be exercised when SIL-mediated hepatoprotective effects in these species are extrapolated to humans. However, the fact that hydroxylating activity was improved by SIL raises the possibility that other hydroxylating systems that, unlike 6β-hydroxylase, are present in humans may be activated by SIL as well. For example, 6α-hydroxylation, which leads to the formation of bile salts that are preferentially glucuronidated and excreted via the urine [26], represents an alternative mechanism to clear out potentially toxic bile acids [27]. Interestingly, rifampicin, which shares with SIL the property of inducing mixed-function oxidases [14], stimulates urinary excretion of glucuronidated 6α-hydroxylated bile salts in human beings.

In conclusion, our results show that SIL administration stimulates bile salt excretion by expanding the endogenous bile salt pool, an effect that is most likely due to an increased formation of CDC as well as its further conversion to hepatoprotective bile salts, including α-MC, β-MC, UDC, and HDC. We hope that these results will encourage further investigation of the changes in bile salt metabolism induced by SIL in humans.

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References

1. Flora K, Hahn M, Rosen H and Benner K, Milk thistle (*Silybum marianum*) for the therapy of liver disease. *Am J Gastroenterol* **93:** 139–143, 1998.
2. Muriel P and Mourelle M, Prevention by silymarin of membrane alterations in acute CCl_4 liver damage. *J Appl Toxicol* **10:** 275–279, 1990.
3. Rauen HM and Schriewer H, The antihepatotoxic effect of silymarin on liver damage in rats induced by carbon tetrachloride, D-galactosamine and allyl alcohol. *Arzneimittelforschung* **21:** 1194–1201, 1971.
4. Muriel P, Garciapina T, Pérez Alvarez V and Mourelle M, Silymarin protects against paracetamol-induced lipid peroxidation and liver damage. *J Appl Toxicol* **12:** 439–442, 1992.
5. Tuchweber B, Sieck R and Trost W, Prevention of phalloidin-induced acute hepatotoxicity. *Toxicol Appl Pharmacol* **51:** 265–275, 1979.
6. Ramellini G and Meldolesi J, Stabilization of isolated rat liver plasma membranes by treatment *in vitro* with silymarin. *Arzneimittelforschung* **24:** 806–808, 1974.
7. Bindoli A, Cavallini L and Siliprandi N, Inhibitory action of Silymarin of lipid peroxide formation in rat liver mitochondria and microsomes. *Biochem Pharmacol* **26:** 2405–2409, 1977.
8. Comoglio A, Leonarduzzi G, Carini R, Busolin D, Basaga H, Albano E, Tomasi A, Poli G, Morazzoni P and Magistretti MJ, Studies on the antioxidant and free radical scavenging properties of IdB 1016, a new flavanolignan complex. *Free Radic Res Commun* **11:** 109–115, 1990.
9. Dehmlow C, Erhard J and De Groot H, Inhibition of Kupffer cell functions as an explanation for the hepatoprotective properties of silibinin. *Hepatology* **23:** 749–754, 1996.
10. Boigk G, Stroedter L, Herbst H, Waldschmidt J, Riecken EO and Schuppan D, Silymarin retards collagen accumulation in early and advanced biliary fibrosis secondary to complete bile duct obliteration in rats. *Hepatology* **26:** 643–649, 1997.
11. Kiesewetter E, Leodolter I and Thaler H, Ergebnisse zweier Doppelblind-studien zur Wirksamkeit von Silymarin bei chronischer Hepatitis. *Leber Magen Darm* **7:** 318–323, 1977.
12. Machicao F and Sonnenbichler J, Mechanism of the stimulation of RNA synthesis in rat liver nuclei by silybin. *Hoppe Seylers Z Physiol Chem* **358:** 141–147, 1977.
13. Magliulo E, Carosi PG, Minoli L and Gorini S, Studies on the regenerative capacity of the liver in rats subjected to partial hepatectomy and treated with silymarin. *Arzneimittelforschung* **23:** 161–167, 1973.
14. Leber HW and Knauff S, Influence of silymarin on drug metabolizing enzymes in rat and man. *Arzneimittelforschung* **26:** 1603–1605, 1976.
15. Hofmann AF and Roda A, Physicochemical properties of bile acids and their relationship to biological properties: An overview of the problem. *J Lipid Res* **25:** 1477–1489, 1984.
16. Vitale GC, Siow Y, Baker PR and Cuschieri A, Reversible bile acid changes in bile duct obstruction and its potential for hepatocellular injury. *J Hepatol* **14:** 151–156, 1992.
17. Lowe PJ and Coleman R, Membrane fluidity and bile salt damage. *Biochim Biophys Acta* **640:** 55–65, 1981.
18. Sokol RJ, Devereaux M, Khandwala R and O'Brien K, Evidence for involvement of oxygen free radicals in bile acid toxicity to isolated rat hepatocytes. *Hepatology* **17:** 869–881, 1993.
19. Kern F Jr, Eriksson H, Curstedt T and Sjövall J, Effect of ethynylestradiol on biliary excretion of bile acids, phosphatidylcholines, and cholesterol in the bile fistula rat. *J Lipid Res* **18:** 623–634, 1977.
20. Schaffner F, Scharnbeck HH, Hutterer F, Denk H, Greim HA and Popper H, Mechanism of α-naphthylisothiocyanate-induced jaundice, *Lab Invest* **28:** 321–331, 1973.

21. Czygan P, Greim H, Trulzsch F, Hutterer F, Schaffner F and Popper H, Alteration in hepatic bile acid patterns in two models of experimental intrahepatic cholestasis. In: *Bile Acids in Human Disease* (Eds. Back P and Gerok W), pp. 135–137. Schattauer, Stuttgart, 1972.
22. Greim H, Trülzsch D, Roboz J, Dressler K, Czygan P, Hutterer F, Schaffner F and Popper H, Mechanism of cholestasis. 5. Bile acids in normal rat livers and in those after bile duct ligation. *Gastroenterology* **63:** 837–845, 1972.
23. Kitani K, Kanai S, Sato Y and Ohta M, Tauro α-muricholate is as effective as tauro β-muricholate and tauroursodeoxycholate in preventing taurochenodeoxycholate-induced liver damage in the rat. *Hepatology* **19:** 1007–1012, 1994.
24. Hofmann AF, Bile acids. In: *The Liver: Biology and Pathobiology* (Eds. Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter D and Shafritz DA), pp. 677–718. Raven Press, New York, 1994.
25. Stiehl A, Rudolph G, Raedsch R, Möller B, Hopf U, Lotterer E, Bircher J, Fölsch U, Klaus J, Endele R and Senn M, Ursodeoxycholic acid-induced changes of plasma and urinary bile acids in patients with primary biliary cirrhosis. *Hepatology* **12:** 492–497, 1990.
26. Nakashima T, Sano A, Seto Y, Nakajima T, Shima T, Sakamoto Y, Okuno T, Kashima K and Hasegawa T, Unusual trihydroxy bile acids in the urine of patients treated with chenodeoxycholate, ursodeoxycholate or rifampicin and those with cirrhosis. *Hepatology* **11:** 255–260, 1990.
27. Wietholtz H, Marschall H-U, Sjövall J and Matern S, Stimulation of bile acid 6α-hydroxylation by rifampin. *J Hepatol* **24:** 713–718, 1996.
28. Borum M and Fromm H, Formation of unusual trihydroxy bile acids in humans: Mechanism for decreasing hydrophobicity and toxic potential of bile acids. *Gastroenterology* **100:** 283–284, 1990.
29. Hardison WGM, Hatoff DR, Miyai K and Weiner RG, Nature of bile acid maximum secretory rate in the rat. *Am J Physiol* **241:** G337–G343, 1981.
30. Accatino L, Hono J, Maldonado M, Icarte MA and Persico R, Adaptive regulation of hepatic bile salt transport. Effect of prolonged bile salt depletion in the rat. *J Hepatol* **7:** 215–223, 1988.
31. Davis RA and Kern F Jr, Effects of ethinylestradiol and phenobarbital on bile acid synthesis and biliary bile acid and cholesterol excretion. *Gastroenterology* 70: 1130–1135, 1976.
32. Roma MG, Luque EA, Marinelli RA and Rodríguez Garay EA, A simple method for the correction of biliary excretion curves distorted by the biliary dead space. *Biochem Pharmacol* **38:** 4021–4025, 1989.
33. Talalay P, Enzymatic analysis of steroid hormones. *Methods Biochem Anal* **8:** 119–143, 1960.
34. Tietz PS, Thiestle JL, Miller LJ and LaRusso NF, Development and validation of a method for measuring the glycine and taurine conjugates of bile acids in bile by high-performance liquid chromatography. *J Chromatogr* **336:** 249–257, 1984.
35. Erlinger S, Bile flow. In: *The Liver: Biology and Pathobiology* (Eds. Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter D and Shafritz DA), pp. 769–786. Raven Press, New York, 1994.
36. Morazzoni P, Magistretti MJ, Giachetti C and Zanolo G, Comparative bioavailability of Silipide, a new flavanolignan complex in rats. *Eur J Drug Metab Pharmacokinet* **17:** 39–44, 1992.
37. Nassuato G, Iemmolo RM, Strazzabosco M, Lirussi F, Deana R, Francesconi MA, Muraca M, Passera D, Fragasso A, Orlando R, Csomós G and Okolicsányi L, Effect of Silibinin on biliary composition. Experimental and clinical study. *J Hepatol* **12**: 290–295, 1991.
38. Carey MC and Duane WC, Enterohepatic circulation. In: *The Liver: Biology and Pathobiology* (Eds. Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter D and Shafritz DA), pp. 719–767. Raven Press, New York, 1994.
39. Lack L and Weiner IM, Intestinal bile acid transport: Structure-activity relationships and other properties. *Am J Physiol* **210**: 1142–1152, 1966.
40. Mosbach EH, Hepatic synthesis of bile acids. Biochemical steps and mechanisms of rate control. *Arch Intern Med* **130:** 478–487, 1972.
41. Delzenne NM, Taper HS and Roberfroid M, Modifications of liver bile acids pool during modulation of rat hepatocarcinogenesis by phenobarbital and nafenopin. *Arch Toxicol* **68:** 394–397, 1994.
42. Vlahcevic ZR, Heuman DM and Hylemon PB, Regulation of bile acid synthesis. *Hepatology* **13:** 590–600, 1991.
43. Roda A, Piazza F, Baraldini M, Speroni E, Guerra MC, Cerré C and Cantelli Forti G, Taurohyodeoxycholic acid protects against taurochenodeoxycholic acid-induced cholestasis in the rat. *Hepatology* **27:** 520–525, 1998.
44. Kitani K, Hepatoprotective properties of ursodeoxycholate in experimental animals. In: *Strategies for the Treatment of Hepatobiliary Disease* (Eds. Paumgartner G, Stiehl A, Barbara L and Roda E), pp. 43–56. Kluwer, Dordrecht, The Netherlands, 1990.