

GALACTOSAMINE PREVENTS ETHINYLESTRADIOL-INDUCED
CHOLESTASIS

Crocenzi, Fernando A.; Pellegrino, José M.; Catania, Viviana A.; Luquita,
Marcelo G., Roma, Marcelo G.; Mottino, Aldo D.; Sánchez Pozzi, Enrique J.

Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y
Farmacéuticas, CONICET – Universidad Nacional de Rosario, Argentina

Running title: Galactosamine prevents ethinylestradiol-induced cholestasis

Author for correspondence:

Dr. Enrique J. Sánchez Pozzi:

Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, CONICET – Universidad Nacional de Rosario. Suipacha 570 (2000) Rosario. Argentina.

Tel: +54-341-4305799

Fax: +54-341-4399473

e-mail: esanchez@unr.edu.ar

Text pages: 11

Tables: 2

Figures: 2

References: 31

Abstract: 220 words

Introduction: 237 words

Discussion: 1198 words

Abbreviations:

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; EE, ethinylestradiol; GAL, galactosamine; SRM, secretory rate maximum; TUDC, tauroursodeoxycholate; UDP-GA, UDP-glucuronic acid.

Abstract

Ethinylestradiol (EE) induces intrahepatic cholestasis in experimental animals, being its derivative ethinylestradiol 17 β -glucuronide a presumed mediator of this effect. To test whether glucuronidation is a relevant step in the pathogenesis of cholestasis induced by EE (5 mg/Kg bw, s.c., for 5 consecutive days), the effect of simultaneous administration of galactosamine (200 mg/kg bw, i.p.) on biliary secretory function was studied. A single injection of this same dose of galactosamine was able to decrease hepatic UDP-glucuronic acid (UDP-GA) levels by 85% and excretion of EE-17 β -glucuronide after administration of a tracer dose of [³H]EE by 40%. Uridine (0.9 g/kg bw, i.p.) coadministration reverted the effect of galactosamine on hepatic UDP-GA levels and restored the excretion of [³H]EE-17 β -glucuronide. When administered for 5 days, galactosamine itself did not alter any of the serum markers of liver injury studied (AST, ALT, ALP) or biliary secretory function. When coadministered with EE, galactosamine partially prevented the impairment induced by this estrogen in total bile flow, the bile salt independent fraction of bile flow, basal bile salt secretion and the secretory rate maximum of tauroursodeoxycholate. Uridine coadministration partially prevented galactosamine from exerting its anticholestatic effects. In conclusion, galactosamine administration partially prevented EE-induced cholestasis by a mechanism involving decreased UDP-GA availability for subsequent formation of EE 17 β -glucuronide. The evidence thus supports the hypothesis that EE 17 β -glucuronide is involved in the pathogenesis of EE cholestasis.

Ethinylestradiol (EE) is a synthetic estrogen that induces intrahepatic cholestasis in experimental animals (Gumucio and Valdivieso, 1971;Bouchard et al., 1993;Jacquemin et al., 1993;Crocenzi et al., 2001). The cholestatic properties of estrogens have been suggested to be mediated by their D-ring glucuronide conjugates, such as estradiol 17 β -glucuronide. (Meyers et al., 1980;Vore, 1987). In contrast, A-ring (3-glucuronide) metabolites are choleric (Meyers et al., 1980). Accordingly, patients with obstetric cholestasis excrete more D- vs. A-ring glucuronides in urine (Adlercreutz et al., 1974), suggesting that D-ring glucuronides may be involved in its pathogenesis. Moreover, in a recent work, our group demonstrated that ursodeoxycholate, a bile salt that prevents estrogen cholestasis, decreases hepatic formation of EE 17 β -glucuronide in the rat (Sanchez Pozzi et al., 2003).

Galactosamine (GAL) is a hepatotoxic drug that markedly depletes hepatic UDP-glucuronic acid (UDP-GA) levels, whereas extrahepatic UDP-GA is minimally affected (Gregus et al., 1988). At a dose of 350 mg/kg bw or higher, GAL induces liver injury, as indicated by a pronounced increase in the serum levels of enzyme markers of hepatic damage, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (Gujral et al., 2003;Markov et al., 1991;Decker and Keppler, 1972). We postulate that, if EE 17 β -glucuronide formation is a relevant step in the pathogenesis of EE-induced cholestasis, a low dose of GAL, enough to decrease UDP-GA synthesis and yet nontoxic, would prevent the alteration in bile flow induced by the estrogen.

MATERIALS AND METHODS

Chemicals. EE, GAL, uridine, UDP-GA, 3 α -hydroxysteroid dehydrogenase, sulfatase type H-1 from *Helix pomatia*, β -glucuronidase type B-1 from bovine liver, NADPH and

DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). Tauroursodeoxycholate (TUDC) was a generous gift from Prodotti Chimici e Alimentari S. p. A. (Genoa, Italy). This bile salt was 99% pure when examined by HPLC. All other reagents were of the highest analytical grade, and used as supplied.

Animals. Adult male Wistar rats weighing 300 to 350 g were used throughout. Animals were maintained on a standard diet and water *ad libitum*, and housed in a temperature- (21°C-23°C) and humidity- (45%-50%) controlled room, under a constant 12-hour light, 12-hour dark cycle. All animals received humane care, according to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the NIH (publication 25-28, revised 1996).

Effect of GAL on hepatic UDP-GA levels. Animals were divided into three groups: *i*) GAL, injected with GAL (a single 200 mg/kg bw dose, i.p.), *ii*) GAL+U, injected with GAL (200 mg/kg bw) and uridine (a single 0.9 g/kg bw dose, i.p.) and *iii*) C, control group, injected with saline, vehicle of GAL and U. Rats were sacrificed by exsanguination under sodium pentobarbital anesthesia 1, 2 or 18 hs after treatment. Livers were quickly excised and placed in liquid nitrogen. A piece of frozen liver was finely ground in a mortar containing liquid nitrogen. One gram of liver powder was homogenized in 20 ml of precooled 150 mM KCl - 10 mM KH₂PO₄ buffer (pH = 7.0). The homogenate was heated in a boiling water bath for 5 min and centrifuged (5000g, 10 min). The supernatant was filtered (0.2 μm, Nalge Nunc International, Rochester, NY) to remove glycogen. A 20-μl aliquot of the filtrate was immediately chromatographed by HPLC, using a 5 μm C18 column. The eluent consisted of methanol-water (50:50 v/v), containing 10 mM KH₂PO₄ and 5 mM tetrabutylammonium hydrogen-sulphate (pH = 7.0). The flow rate was 1 ml/min. UDP-GA was monitored by UV absorption at 260 nm (Alary et al., 1992).

Effect of GAL on biliary excretion of EE metabolites. Animals of the same three groups described above were anaesthetized with a single dose of sodium pentobarbital (50 mg/kg bw, i.p.), and both the femoral vein and the common bile duct were cannulated with a PE-50 and PE-10 polyethylene tubing, respectively (Intramedic, Clay Adams, Parsippany, NJ). Two hours after GAL administration, a tracer dose of [³H]EE (10 µCi/kg bw) was injected *via* the femoral vein. Immediately, bile was collected in 20-min intervals for 60 min. At the end of the experiment, the animals were sacrificed and livers removed and weighed. Total radioactivity in bile and glucuronide and sulfate fractions of EE were measured as described (Sanchez Pozzi et al., 2003).

Effect of GAL on biliary secretory function. Animals were divided into 6 experimental groups:

- i) **Control**, receiving the vehicle of EE, propylene glycol (0.5 ml/kg bw, s.c.), and the vehicle of both GAL and uridine, saline (0.5 ml/kg bw, i.p.), for 5 consecutive days.
- ii) **EE**, administered daily with EE (5 mg/kg bw, s.c.) and saline, for 5 days.
- iii) **EE+GAL**, co-administered daily with EE, GAL (200 mg/kg bw, i.p.), and saline for 5 days.
- iv) **EE+GAL+U**, co-administered daily with EE, GAL, and uridine (0.9 g/kg bw, i.p.) for 5 days.
- v) **GAL**, co-administered daily with GAL, and propylene glycol for 5 days.
- vi) **U**, co-administered daily with uridine, and propylene glycol for 5 days.

Surgical procedures were made on the sixth day, i.e. one day after administration of the last EE dose. Bile collection started between 9:00 a.m. and 11:00 a.m. to minimize influence of circadian variations on bile flow. Animals were anaesthetized, and maintained under this condition throughout the experiment. A middle abdominal incision was made, and the common bile duct cannulated using a PE-10 polyethylene

tubing. Tracheal cannulation was systematically performed to remove bronchial secretion induced by the anesthetic. Body temperature was maintained at 37.0°C-38.5°C with a warming lamp.

Basal bile secretion studies. Thirty min after bile duct cannulation, bile was collected for two 15-min periods, one of which was collected in tubes containing 1 ml of 6% 5-sulfosalicylic acid for total glutathione (reduced+oxidized) determination. To evaluate biliary HCO_3^- secretion, basal bile was collected under liquid Vaseline™ to avoid equilibration with atmospheric CO_2 . At the end of bile collection, the animals were sacrificed by exsanguination, and the livers removed and weighed. Bile flow was determined by gravimetry, assuming a bile density of 1.0 g/ml. Basal bile was assayed for total and individual bile salt content. Total bile salt concentration was assessed using the 3α -hydroxysteroid dehydrogenase procedure (Talalay, 1960). Individual bile salts were determined by HPLC (Waters, Mildford, MA), as reported (Tietz et al., 1984), using authentic standards. Total glutathione was measured by the recycling method of Tietze (Tietze, 1969). HCO_3^- concentration in bile was measured immediately after bile collection in an automated blood-gas analyzer (Compact 1, AVL Medical Instruments AG). Biliary excretion rates were calculated as the product between bile flow and biliary concentration. Activities of the enzyme markers of hepatocellular injury, AST (EC 2.6.1.1), ALT (EC 2.6.1.2) and ALP (EC 3.1.3.1), were determined in plasma using commercial kits (Wiener Lab, Rosario, Argentina).

TUDC Infusion Studies. Rats from the same 6 experimental groups were infused with TUDC to determine the secretory rate maximum (SRM) of this bile salt into bile. TUDC was chosen due to its extremely low toxicity, in contrast to other naturally occurring bile salts, whose apparent maximum transport is largely dependent on their cytotoxicity rather than by saturation of their canalicular transport system (Hardison et al., 1981).

The SRM of TUDC was assessed by infusing the bile salt intravenously, dissolved in 2% BSA in saline, at stepwise-increasing rates (2.0, 2.5, 5.0, 6.5, 12.0 and 16.0 $\mu\text{mol}/\text{min}/100\text{g}$ body wt). Each infusion rate was maintained for 20 min, and bile samples were collected every 10 min for 120 min. SRM was calculated as the mean of the three highest, consecutive, secretory rates recorded over the whole infusion period (Hardison et al., 1981). The bile-salt-independent fraction of bile flow (BSIF) was estimated in these animals by the conventional extrapolation to zero bile salt output of the regression line between bile flow and bile salt output, as stimulated by TUDC infusion (Erlinger, 1994).

Statistical Analysis. Results were expressed as mean \pm SE. One way ANOVA, followed by Newman-Keuls test, were performed for multiple comparison among groups. The regression line analysis was done by the least square method. The significance of the differences between slopes and between y-intercepts was assayed by covariance analysis. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Effect of GAL on UDP-GA levels.

A single dose of GAL reduced hepatic UDP-GA content by 85% two hours after its injection, being this reduction fully prevented by uridine administration (Fig 1). Eighteen hours after treatment, UDP-GA levels return to control values.

Biliary excretion of EE metabolites.

Two hours after injection of [^3H]EE, cumulative biliary excretion of total [^3H] decreased approximately by 10 % in GAL treated-rats (C: $74 \pm 2\%$, GAL: $66 \pm 2\%$ of the injected dose, $p < 0.05$) and was restored to C values by U (GAL+U: $75 \pm 3\%$ of the injected dose, $p < 0.05$ vs GAL). A comparative HPLC analysis of untreated bile

samples vs. samples preincubated with β -glucuronidase or sulfatase + β -glucuronidase, led to the identification of one peak as a sulfate derivative, and other two as glucuronides. One of the glucuronide peaks was identified as EE 17 β -glucuronide. GAL treatment produced a decrease of approximately 40% in the excretion of this compound (C: 5.9 \pm 0.6%, GAL: 3.4 \pm 0.4% of the injected dose, $p < 0.05$), which was restored by U coadministration (GAL+U: 5.8 \pm 0.5%; $p < 0.05$ vs GAL). The excretion of the other glucuronide was also decreased by GAL (C: 13.8 \pm 1.3%, GAL: 8.2 \pm 0.9%, of the injected dose, $p < 0.05$), and restored to C values by U administration (GAL+U: 14.0 \pm 1.4% $p < 0.05$ vs GAL). The excretion of the sulfate derivative was not modified by the treatments (C: 18 \pm 2%, GAL: 20 \pm 2%, GAL+U: 21 \pm 2%).

Serum markers of hepatocellular integrity.

As shown in Table 1, neither ALT nor AST was affected by GAL administration, indicating that the protocol used to study GAL action on biliary secretory function was not toxic. Neither of these serum markers was modified by EE, in agreement with previous results (Bouchard et al., 1993;Crocenzi et al., 2001;Sanchez Pozzi et al., 2003). However, EE treatment led to a 40% increase in ALP serum activity, a biochemical marker of cholestasis (Table 1), which was not restored by GAL coadministration.

Effect of GAL on basal bile flow and bile salt output.

Table 1 shows that GAL partially prevented EE-induced reduction in bile flow, whereas uridine coadministration blocked this preventive effect. Table 1 also shows that conjoint EE and GAL treatment partially restored the excretion of the individual bile salts decreased by EE, with excretion rates of cholate and chenodeoxycholate reaching statistical significance. Uridine coadministration blocked the protective effect of GAL only for cholate excretion. The counteracting effect of uridine was not evident for total

bile salt secretion, most probably due to a non-significant increase in the biliary excretion of muricholate, the main biliary bile salt in the rat.

Effect of GAL on the impairment of the hepatic handling of TUDC induced by EE.

Table 2 shows the effect of the different treatments on SRM of the non-toxic bile salt TUDC. This parameter was diminished by EE treatment (-88%), and partially restored by GAL coadministration (+125% as compared with EE group). Uridine coadministration abolished GAL effects.

Effect of GAL on the decrease of the BSIF induced by EE.

As can be seen in Fig. 2, a significant decrease in BSIF, as estimated by the y-intercept of the regression line between bile flow and bile salt output under TUDC infusion, was found in EE-treated rats (0.56 $\mu\text{l}/\text{min}/\text{g}$ liver wt versus 1.93 $\mu\text{l}/\text{min}/\text{g}$ liver wt in Control; $p < 0.05$). GAL partially prevented this impairment (1.11 $\mu\text{l}/\text{min}/\text{g}$ liver wt, $p < 0.05$ vs. EE) and U coadministration blocked GAL effect (0.69 $\mu\text{l}/\text{min}/\text{g}$ liver wt, $p < 0.05$ vs. GAL). None of the treatments modified the slope of the regression lines, which represents the choloretic efficiency of the secreted bile salts.

To characterize the mechanism(s) involved in the decrease of BSIF induced by EE and its further prevention by GAL, biliary excretion of total glutathione and HCO_3^- , compounds thought to be the major driving forces for BSIF (Ballatori and Truong, 1992; Hardison and Wood, 1978), were assessed. As can be seen in Table 1, basal biliary excretions of both glutathione and HCO_3^- were significantly diminished by EE pretreatment. Impairment in the biliary excretion of HCO_3^- was significantly but only partially prevented by GAL. Although there was a trend toward increased glutathione output in the EE-treated group receiving also GAL, the differences were not statistically significant; nevertheless, the eventual recovery of this parameter was insignificant compared to control values.

DISCUSSION

EE is a synthetic estrogen known to induce intrahepatic cholestasis in animals. Conversion of EE in specific metabolite/s seems to be implicated in its cholestatic action since impairment of bile flow after estrogen administration takes several hours to be detected (Gumucio and Valdivieso, 1971). Some evidences point towards EE 17 β -glucuronide as the putative mediator. Indeed, this and other D-ring glucuronide conjugates, such as estradiol 17 β -glucuronide, induce acute cholestasis (Meyers et al., 1980; Vore, 1987). Furthermore, in a previous work, we demonstrated that ursodeoxycholate, a bile salt that prevents EE cholestasis, decreases the formation of the 17 β -glucuronide derivative of EE (Sanchez Pozzi et al., 2003).

In the present work, we provide further, more compelling evidence to support the role of glucuronidation in the development of EE cholestasis. We administered GAL, a compound that selectively decreases hepatocyte UDP-GA levels, and found a protective effect in cholestasis. Due to its toxic nature, it was important to rule out any harmful effect of GAL at the dose administered here. Indeed, at doses of 350 mg/kg bw or higher, this compound produces hepatotoxicity, as a consequence of the decrease in mRNA synthesis due to shortage of uridine nucleotides. This decrease is fully reverted by the administration of uridine (Keppler et al., 1974). In our experiments, we used a daily dose of 200 mg/kg bw, in a compromise between UDP-GA depletion and toxicity. Serum levels of the enzyme markers of hepatotoxicity (ALT and AST) were not affected by this dose of GAL, excluding overt toxicity in our protocol. In addition, none of the functional parameters evaluated such as bile flow, biliary excretions of bile salts and glutathione, or SRM of TUDC was modified by GAL administration. However, this dose was able to produce a partial decrease (-85%) in hepatic UDP-GA content, and

concomitant decrease in biliary excretion of EE glucuronides by 40%. Because production of estrogen glucuronide was not totally abolished, a remaining cholestatic effect of EE in GAL co-treated rats was expected. Uridine co-administration, which restored hepatic UDP-GA level, reverted, totally or partially, the effects of GAL on bile flow, SRM of TUDC and BSIF, confirming that shortage of UDP-GA was responsible for GAL effects.

Impairment of bile salt secretion by EE can be attributed to both diminution of the bile salt pool size (Crocenzi et al., 2001) and diminished expression of the canalicular bile salt export pump (Bsep) (Lee et al., 2000). The quantitative and qualitative alterations induced by EE in the bile salt pool are complex and likely result from simultaneous inhibition of the activity of microsomal cholesterol 7- α -hydroxylase (Cyp7a1) (Koopen et al., 1999; Davis et al., 1986), and preservation of the mitochondrial sterol 27-hydroxylase (Cyp27a1) (Kern et al., 1977). Cyp7a1 is the enzyme that catalyses the key, rate-limiting step of the *classical*, major pathway of the overall bile salt synthesis, whereas Cyp27a1 catalyses the first step in the *alternative* pathway of bile salt synthesis, which produces selectively chenodeoxycholate and the bile salts derived from its further hepatic and/or intestinal conversion, *i.e.*, muricholate and hiodeoxycholate (Koopen et al., 1999). These metabolic effects led to a preservation of the biliary excretion of both muricholate and hiodeoxycholate, and a decrease in the biliary excretion of the other bile salts by the estrogen. Our finding that GAL reverted this effect of EE, showing a control-like pattern of biliary bile salt output, suggests that EE-glucuronide can be involved in these metabolic effects of EE.

Experiments performed with infusion of TUDC revealed that GAL also prevented, though to a lower extent, the alteration in Bsep transport activity induced by EE, thus implicating, at least partially, EE-17 β -glucuronide as a mediator. It is well

accepted that SRM estimates the total number of active transporters at the canalicular membrane. Diminution of Bsep activity by EE may result from an inhibitory action of cholestatic derivatives of EE, as was observed with estradiol 17 β -glucuronide (Stieger et al., 2000), or alternatively, from a reduction in Bsep protein expression (Lee et al., 2000). Either or both of these factors may indeed explain the decreased SRM of TUDC in EE group, as previously reported (Crocenzi et al., 2001). The mechanisms involved in Bsep expression decrease are poorly understood; acute administration of the endogenous estrogen derivative, estradiol-17 β -glucuronide, was reported to produce a rapid and reversible endocytic internalization of Bsep, with concomitant reduction in activity (Crocenzi et al., 2003). Persistent exposure to estrogen cholestatic metabolites, like EE 17 β -glucuronide, as can be expected to occur following sub-acute administration of EE, may lead to delivery of Bsep-containing vesicles to the lysosomal compartment, followed by lysosomal degradation; this phenomenon was hypothesized to be responsible for Mrp2 downregulation several hours after endotoxin administration (Kubitz et al., 1999) and after bile duct ligation (Paulusma et al., 2000).

As previously reported, EE impaired BSIF by diminishing the biliary excretion of glutathione (Bouchard et al., 1994) and HCO₃⁻ (Alvaro et al., 1997), two compounds thought to be the main responsible for this fraction of bile flow. Impairment of BSIF by EE was also partially protected by GAL, which reverted the effects of the estrogen on HCO₃⁻ excretion but not on glutathione output. Since western blot analysis demonstrated that AE2, the canalicular transporter of HCO₃⁻, is preserved in EE cholestasis (Alvaro et al., 1997), a functional alteration of the transporter could be responsible for HCO₃⁻ excretion impairment. GAL prevention of estrogen-induced alteration in HCO₃⁻ excretion suggests that EE-glucuronide could participate in the alleged functional alteration of the transporter, though the mechanism remains

unknown. Our finding that GAL was not effective in preventing glutathione secretory failure indicates that alteration of glutathione excretion induced by EE could be independent of EE-glucuronide formation.

In conclusion, GAL administration partially prevented EE cholestasis *via* a mechanism that involves a decrease in UDP-GA availability. The consequent decrease in EE 17 β -glucuronide formation most likely accounted for this prevention, supporting the hypothesis that EE 17 β -glucuronide plays a key role in the cholestasis induced by its unconjugated, parent compound.

References

- Adlercreutz H, Tikkanen MJ, Wichmann K, Svanborg A, and Anberg A (1974) Recurrent jaundice in pregnancy. IV. Quantitative determination of urinary and biliary estrogens, including studies in pruritus gravidarum. *J Clin Endocrinol Metab* 38:51-57.
- Alary J, Cravedi JP, Baradat M, and Carrera G (1992) Mechanism of cadmium-decreased glucuronidation in the rat. *Biochem Pharmacol* 44:2139-2147.
- Alvaro D, Gigliozzi A, Piat C, Carli L, Fraioli F, Romeo R, Francia C and et al. (1997) Inhibition of biliary bicarbonate secretion in ethinylestradiol-induced cholestasis is not associated with impaired activity of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the rat. *J Hepatol* 26:146-157.
- Ballatori N and Truong AT (1992) Glutathione as a primary osmotic driving force in hepatic bile formation. *Am J Physiol* 263:G617-G624.
- Bouchard G, Yousef IM, and Tuchweber B (1993) Influence of oral treatment with ursodeoxycholic and tauroursodeoxycholic acids on estrogen-induced cholestasis in rats: effects on bile formation and liver plasma membranes. *Liver* 13:193-202.
- Bouchard G, Yousef IM and Tuchweber B (1994) Decreased biliary glutathione content is responsible for the decline in bile salt-independent flow induced by ethinylestradiol in rats. *Toxicol Lett* 74:221-233.
- Crocenzi FA, Sánchez Pozzi EJ, Pellegrino JM, Favre CO, Rodríguez Garay EA, Mottino AD, Coleman R, and Roma MG (2001) Beneficial effects of silymarin on estrogen-induced cholestasis in the rat: a study in vivo and in isolated hepatocyte couplets. *Hepatology* 34:329-339.

- Crocenzi FA, Mottino AD, Cao J, Veggi LM, Sanchez Pozzi EJ, Vore M, Coleman R and Roma MG (2003) Estradiol-17- β D-glucuronide induces endocytic internalization of Bsep in rats. *Am J Physiol Gastrointest Liver Physiol* 285:G449-G459.
- Davis RA, Elliott TS, Lattier GR, Showalter RB and Kern FJr (1986) Regulation of bile acid synthesis via direct effects on the microsomal membrane. *Biochemistry* 25:1632-1636.
- Decker K and Keppler D (1972) Galactosamine induced liver injury. *Prog Liver Dis* 4:183-199.
- Erlinger S (1994) Bile flow, in *The liver: Biology and Pathobiology* (Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, and Shafritz DA eds) pp 769-786, Raven Press, New York.
- Gregus Z, Madhu C, Goon D, and Klaassen CD (1988) Effect of galactosamine-induced hepatic UDP-glucuronic acid depletion on acetaminophen elimination in rats. Dispositional differences between hepatically and extrahepatically formed glucuronides of acetaminophen and other chemicals. *Drug Metab Dispos.* 16:527-533.
- Gujral JS, Farhood A, and Jaeschke H (2003) Oncotic necrosis and caspase-dependent apoptosis during galactosamine-induced liver injury in rats. *Toxicol Appl Pharmacol* 190:37-46.
- Gumucio JJ and Valdivieso VD (1971) Studies on the mechanism of the ethynylestradiol impairment of bile flow and bile salt excretion in the rat. *Gastroenterology* 61:339-344.

- Hardison WG and Wood CA (1978) Importance of bicarbonate in bile salt independent fraction of bile flow. *Am J Physiol* 235:E158-E164.
- Hardison WG, Hatoff DE, Miyai K, and Weiner RG (1981) Nature of bile acid maximum secretory rate in the rat. *Am J Physiol* 241:G337-G343.
- Jacquemin E, Dumont M, Mallet A, and Erlinger S (1993) Ursodeoxycholic acid improves ethinyl estradiol-induced cholestasis in the rat. *Eur J Clin Invest* 23:794-802.
- Keppler DO, Pausch J, and Decker K (1974) Selective uridine triphosphate deficiency induced by D-galactosamine in liver and reversed by pyrimidine nucleotide precursors. Effect on ribonucleic acid synthesis. *J Biol Chem* 249:211-216.
- Kern FJr, Eriksson H, Curstedt T and Sjövall J (1977) Effect of ethinylestradiol on biliary excretion of bile acids, phosphatidylcolines, and cholesterol in the bile fistule rat. *J Lipid Res* 18:623-634.
- Koopen NR, Post SM, Wolters H, Havinga R, Stellaard F, Boverhof R, Kuipers F and Princen HMG (1999) Differential effects of 17 α -ethinylestradiol on the neutral and acidic pathways of bile salt synthesis in the rat. *J Lipid Res* 40:100-108.
- Kubitz R, Wettstein M, Warskulat U and Häussinger D (1999) Regulation of the multidrug resistance protein 2 in the rat liver by lipopolysaccharide and dexamethasone. *Gastroenterology* 116:401-410.
- Lee JM, Trauner M, Soroka CJ, Stieger B, Meier PJ and Boyer JL (2000) Expression of the bile salt export pump is maintained after chronic cholestasis in the rat. *Gastroenterology* 118:163-172.

Markov AK, Farias LA, Bennett WS, Subramony C, and Mihas AA (1991) Prevention of galactosamine-induced hepatotoxicity in rats with fructose-1,6-diphosphate.

Pharmacology 43:310-317.

Meyers M, Slikker W, Pascoe G, and Vore M (1980) Characterization of cholestasis induced by estradiol-17 beta-D-glucuronide in the rat. *J Pharmacol Exp Ther*

214:87-93.

Paulusma CC, Kothe MJ, Bakker CT, Bosma PJ, van Bokhoven I, van Marle J, Bolder U, Tytgat GN, Oude Elferink RP (2000) Zonal down-regulation and redistribution of the multidrug resistance protein 2 during bile duct ligation in rat liver.

Hepatology 31:684-693

Sanchez Pozzi EJ, Crocenzi FA, Pellegrino JM, Catania VA, Luquita MG, Roma MG, Rodriguez Garay EA, and Mottino AD (2003) Ursodeoxycholate reduces ethinylestradiol glucuronidation in the rat: role in prevention of estrogen-induced cholestasis. *J Pharmacol Exp Ther* 306:279-286.

Stieger B, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. (2000) Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* 118:422-30.

Talalay P (1960) Enzymatic analysis of steroid hormones. *Methods Biochem Anal* 8:119-143.

Tietz PS, Thistle JL, Miller LJ, and LaRusso NF (1984) 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.

Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 27:502-522.

Vore M (1987) Estrogen cholestasis. Membranes, metabolites, or receptors? *Gastroenterology* 93:643-649.

Footnotes

Supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), ARGENTINA.

Parts of this study were presented in the Biannual Meeting of the International Association for the Study of the Liver (IASL, San Salvador de Bahia, Brazil), March 2004.

Reprint request to:

Dr. Enrique J. Sánchez Pozzi:

Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, CONICET – Universidad Nacional de Rosario. Suipacha 570 (2000) Rosario. Argentina.

Tel: +54-341-4305799

Fax: +54-341-4399473

e-mail: esanchez@unr.edu.ar

Legends for figures

Fig 1. Hepatic UDP-GA levels.

UDP-GA levels were determined by HPLC in liver homogenates from Control rats and from rats injected with galactosamine (200 mg/kg bw, GAL), or galactosamine plus uridine (0.9 g/kg bw, GAL+U), 1, 2 or 18 hs after injection. All values are mean \pm SE of 3 animals per group.

^a Different from Control and GAL 1 h ($p < 0.05$); ^b different from GAL 2 h ($p < 0.05$); ^c different from Control, GAL 1h, GAL 2h and GAL+U 1h ($p < 0.05$).

*Fig 2. Relationship between bile flow and bile salt output, as stimulated by a steady-step infusion of TUDC, in animals pre-treated with ethinylestradiol alone (5 mg/kg body wt per day for 5 days, s.c., EE, ∇), or in combination with galactosamine (200 mg/kg bw per day for 5 days, i.p., EE+GAL, \blacksquare) or galactosamine+uridine (0.9 g/kg bw per day for 5 days, i.p., EE+GAL+U, \square). Animals receiving only galactosamine (GAL, \circ), uridine (U, \blacktriangledown) or controls receiving only the vehicle (propylene glycol, Control, \bullet) are also shown. Regression analysis yielded the following equations: **Control:** $y = 0.0046 \pm 0.0002 x + 1.94 \pm 0.06$; **GAL:** $y = 0.0046 \pm 0.0001 x + 1.80 \pm 0.04$; **U:** $y = 0.0049 \pm 0.0002 x + 1.76 \pm 0.10$; **EE:** $y = 0.0043 \pm 0.0005 x + 0.56 \pm 0.05$; **EE+GAL:** $y = 0.0041 \pm 0.0003 x + 1.11 \pm 0.03$; **EE+GAL+U:** $y = 0.0041 \pm 0.0005 x + 0.69 \pm 0.04$. The correlation coefficient was > 0.88 for all groups.*

Table 1. Effect of galactosamine administration on alterations of bile flow, bile salt output and serum markers induced by EE.

	Control	GAL	U	EE	EE+GAL	EE+GAL+U
Bile Flow (µl/min/g liver)	2.29±0.14	2.13±0.05	2.24±0.16	0.90±0.05 ^a	1.35±0.08 ^{a,b}	0.99±0.07 ^{a,c}
Bile salt output (nmol/min/g liver)	51.9±3.6	57±3.9	59.2±3.7	28.1±1.6 ^a	42.2±2.4 ^{a,b}	37.7±3.9 ^{a,b}
Total glutathione output (nmol/min/g liver)	2.80±0.02	2.88±0.11	3.01±0.11	0.05±0.01 ^a	0.11±0.04 ^a	0.02±0.01 ^a
HCO ₃ ⁻ output (nmol/min/g liver)	50±3	48±1	52±3	20±3 ^a	30±2 ^{a,b}	19±2 ^{a,c}
Individual bile salt output (nmol/min/g liver)						
MC	23.2±2.7	27.1±2.6	27.7±2.5	18.8±1.6	20.6±2.1	23.7±2.2
UDC	2.8±0.5	3.8±0.6	3.7±0.7	0.8±0.2 ^a	2.0±0.3	1.2±0.3 ^a
HDC	3.2±0.6	3.0±0.4	3.3±0.3	2.2±0.4	3.3±0.4	2.9 ± 0.4
C	17.3±1.3	18.2±2.2	19.9±1.3	5.5±0.7 ^a	13.0±0.9 ^{a,b}	8.1±1.2 ^{a,c}
CDC	3.6±0.5	2.4±0.5	2.4±0.6	0.3±0.1 ^a	2.0±0.4 ^b	1.2±0.4 ^a
DC	1.9±0.3	2.6±0.4	2.2±0.3	0.4±0.2 ^a	1.3±0.2	0.6±0.2 ^a
Serum biochemical parameters						
AST (U/L)	135±29	72±21	132±25	167±24	98±16	99±15
ALT (U/L)	138±16	77±18	134±21	131±13	84±21	94±25
ALP (U/L)	298±32	179±38	285±28	416±29 ^a	405±19 ^a	400±21 ^a

All parameters were assessed after a 5-day treatment with ethinylestradiol (EE, 5 mg/kg bw), galactosamine (GAL, 200 mg/kg bw), uridine (U, 0.9 g/kg bw), EE+GAL, EE+GAL+U, or vehicle in Control group. Bile salts were analyzed by HPLC, as described in Materials and Methods, in basal bile collected for 15 min. Results are expressed as mean ± SE for 8-12 animals per group. ^a Significantly different from Control ($p<0.05$); ^b significantly different from EE ($p<0.05$); ^c significantly different from EE+GAL ($p<0.05$). Abbreviations: MC, muricholate; UDC, ursodeoxycholate; HDC, hiodeoxycholate; C, cholate; CDC, chenodeoxycholate; DC, deoxycholate.

Table 2. Effect of GAL on the alterations induced by EE on SRM of TUDC

	SRM of TUDC (nmol/min/g liver wt)
Control	898±27
GAL	825±77
U	945±35
EE	112±16 ^a
EE+GAL	252±18 ^{ab}
EE+GAL+U	139±11 ^a

SRM of TUDC was determined in animals pretreated with a combination of a daily dose of GAL (200 mg/kg bw, i.p., for 5 days), EE (5 mg/kg bw, s.c., for 5 days), uridine (0.9 g/kg bw, i.p., for 5 days), or vehicle (propylene glycol). SRM values were calculated as the mean of the 3 highest, consecutive 10-min biliary secretory rates recorded over the whole TUDC infusion periods.

Results are expressed as mean ± SE for 4 animals per group.

^a Different from Control group ($p < 0.05$); ^b Different from EE group ($p < 0.05$).

Fig 1



