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ORIGINAL ARTICLE

Hormonal Modulation of Hepatic cAMP Prevents Estradiol 17β-D-Glucuronide-Induced Cholestasis in Perfused Rat Liver

Andrés E. Zucchetti · Ismael R. Barosso · Andrea C. Boaglio · Marcelo G. Luquita · Marcelo G. Roma · Fernando A. Crocenzi · Enrique J. Sánchez Pozzi

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Abstract
Background Estradiol-17β-D-glucuronide (E17G) induces cholestasis in vivo, endocytic internalization of the canalicular transporters multidrug resistance-associated protein 2 (Abcc2) and bile salt export pump (Abcb11) being a key pathomechanism. Cyclic AMP (cAMP) prevents cholestasis by targeting these transporters back to the canalicular membrane. In hepatocyte couplets, glucagon and salbutamol, both of which increase cAMP, prevented E17G action by stimulating the trafficking of these transporters by different mechanisms, namely: glucagon activates a protein kinase A-dependent pathway, whereas salbutamol activates an exchange-protein activated by cAMP (Epac)-mediated, microtubule-dependent pathway.
Methods The present study evaluated whether glucagon and salbutamol prevent E17G-induced cholestasis in a more physiological model, i.e., the perfused rat liver (PRL). Additionally, the preventive effect of in vivo alanine administration, which induces pancreatic glucagon secretion, was evaluated.
Results In PRLs, glucagon and salbutamol prevented E17G-induced decrease in both bile flow and the secretory activity of Abcc2 and Abcb11. Salbutamol prevention fully depended on microtubule integrity. On the other hand, glucagon prevention was microtubule-independent only at early time periods after E17G administration, but it was ultimately affected by the microtubule disrupter colchicine. Cholestasis was associated with endocytic internalization of Abcb11 and Abcc2, the intracellular carriers being partially colocalized with the endosomal marker Rab11a. This effect was completely prevented by salbutamol, whereas some transporter-containing vesicles remained colocalized with Rab11a after glucagon treatment. In vivo, alanine administration increased hepatic cAMP and accelerated the recovery of bile flow and Abcb11/Abcc2 transport function after E17G administration. The initial recovery afforded by alanine was microtubule-independent, but microtubule integrity was required to sustain this protective effect.
Conclusion We conclude that modulation of cAMP levels either by direct administration of cAMP modulators or by physiological manipulations leadings to hormone-mediated increase of cAMP levels (alanine administration), prevents estrogen-induced cholestasis in models with preserved liver architecture, through mechanisms similar to those arisen from in vitro studies.

Keywords Glucagon · Salbutamol · Abcc2 · Abcb11 · Rab11a · Microtubule

Abbreviations

Abcb11	Bile salt export pump
Abcc2	Multidrug-resistance-associated protein 2
Ala	Alanine
E17G	Estradiol 17β-D-glucuronide
DNP-G	S-(2,4-Dinitrophenyl)-glutathione
TC	Sodium taurocholate
Sal	Salbutamol
Glu	Glucagon
DMSO	Dimethyl sulfoxide
PRL	Perfused rat liver

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Introduction

Bile formation relies on the coordinated action of a number of transporters in the hepatocyte sinusoidal and canalicular domains. Transport of solutes through the canalicular membrane is the rate-limiting step in bile formation, and depends on the normal activity of ATP-dependent transporters belonging to the ATP binding cassettes (ABC) superfamily [1, 2]. Among the most relevant, there are the bile salt export pump (Abcb11, also named Bsep), which transports monoanionic bile salts, and the multidrug resistance-associated protein 2 (Abcc2, also named Mrp2), which transports glutathione and glutathione conjugates, as well as a wide variety of anionic compounds, including sulfated or glucuronidated bile salts and bilirubin mono- and di-glucuronides [1, 2]. Since bile salts and glutathione are chief determinants of the so-called bile salt-dependent and bile salt-independent fractions of the bile flow, respectively [3], alterations in activity, localization and/or expression of their respective transporters, Abcb11 and Abcc2, lead to cholestasis [4, 5].

The levels of D-ring metabolites increase during pregnancy [7] and may be a key factor in the pathogenesis of intrahepatic cholestasis occurring in pregnant, susceptible women [6]. Estradiol-17β-D-glucuronide (E17G) is a D-ring endogenous metabolite of estradiol that induces an acute and reversible cholestasis in vivo, by impairing both fractions of bile flow [6]. The mechanisms by which D-ring metabolites induce cholestasis seem to be multifactorial. Trans-inhibition of Bsep-mediated canalicular transport of bile salts by these metabolites [8, 9] has been shown to be a causal factor. In addition, our group recently showed that endocytic internalization of both Abcb11 [4] and Abcc2 [10, 11] is also a key cholestatic mechanism of E17G. Furthermore, it is becoming increasingly recognized that canalicular transporter internalization represents a feature common to many other cholestatic conditions both in experimental animals and in humans [12]. Under cholestatic conditions, Abcb11 and Abcc2 leave the canalicular membrane by undergoing endocytic internalization into vesicular compartments, a phenomenon systematically associated with failure in the secretion of their specific substrates. Under normal conditions, there is a balance between endocytic internalization and exocytic insertion of transporters. Since cholestasis disturbs this equilibrium towards internalization, it is conceptually reasonable to hypothesize that signaling molecules that stimulate routes of exocytic insertion can restore the normal balance. Also, the exocytic reinsertion of the endocytosed transporters that spontaneously occurs after the initial cholestatic insult with E17G [4] can be accelerated by these signaling molecules, thus providing a mean to speed up recovery from cholestasis. One signaling mediator bearing such stimulatory properties is cAMP. In liver, this signaling messenger stimulates the trafficking of hepatocellular transporters from their synthesis sites to the canalicular membrane in a microtubule-dependent manner, as well as the fusion of transporter-containing vesicles from a subapical compartment with the apical membrane [13, 14]. In recent works, our group demonstrated that cAMP prevents the internalization and accelerates the re-insertion of ABC transporters in E17G-induced cholestasis [4, 10].

cAMP is the second messenger of a number of hormones that act at the hepatic level, such as glucagon (Glu), vasoactive intestinal peptide, and catecholamines [15]. Among the hormones with potential anticholestatic properties, Glu and adrenaline may be assumed as the most appropriate ones. Glucagon is recognized for its capability to both promote bile flow [16] and elevate hepatic cAMP levels in humans [15]. In addition, Glu is produced in the pancreas and delivered into portal circulation, thus reaching the liver at high levels before entering the systemic circulation. Adrenaline increases hepatic cAMP levels via the β2 adrenergic receptor [17]. It shares with Glu some cAMP-dependent hormonal effects in liver, such as stimulation of glycogenolysis [18]. Consequently, any change in synthesis or release of these hormones is expected to heavily impact the liver in terms of signaling.

Recently, we have demonstrated, in isolated rat hepatocytes couplets, an in vitro polarized model for the study of hepatocanalicular transport function, that both Glu and the β2 adrenergic receptor agonist salbutamol (Sal) prevent the canalicular transporter delocalization induced by E17G [19]. Surprisingly, the intracellular mechanisms underlying protection by cAMP differ when triggered by each of these agents. Glu activates a PKA-mediated, microtubule-independent pathway, whereas Sal activates an Epac-MEK-dependent pathway that requires an intact microtubular network. Moreover, confocal images indicate that the pool of transporter-containing vesicles implicated in the reinsertion process is different in each case. E17G induces internalization of transporters into endosomal vesicles, since they colocalize with Rab11a, an endosomal marker. Sal fully reinserts transporters, whereas Glu leaves some transporters colocalized with the endosomal marker in the perinuclear region. Overall, this indicates the existence of two different pools of transporters, namely the ones that are reinserted by Glu from underneath the canalicular membrane and the ones mobilized by Sal from a deeper compartment with the aid of microtubules.

The aim of the present work was to evaluate the capability of the cAMP-elevating agonists, Glu and Sal, to prevent E17G-induced cholestasis in a more physiological model, such as the perfused rat liver (PRL). This model allows the evaluation of the time course of the protection, information useful to determine whether these compounds counteract the triggering of cholestasis or whether they accelerate the recovery phase after the initial insult. Additionally, since alanine (Ala) administration is known

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to elevate hepatic cAMP presumably through pancreatic Glu release, we evaluated the potential protective effect of this amino acid in E17G-induced cholestasis in vivo.

Materials and Methods

Chemicals

E17G, Sal, (TC), Ala, 1-chloro-2,4-dinitrobenzene (CDNB), 3-α hydroxysteroid dehydrogenase, bovine serum albumin (BSA), dimethylsulfoxide (DMSO), NAD, Triton X-100 and colchicine were from Sigma Chemical Co. (St. Louis, MO, USA). Glu was from Eli Lilly and Co. (Indianapolis, IN, USA). Rabbit anti-rat Abcb11 was from Kamiya Biomedical Co. (Seattle, WA, USA). Mouse anti-human Abcc2 (M2III-6) was from Alexis Biochemicals (San Diego, CA, USA). Mouse anti-human Rab11a was from Abcam (Cambridge, MA, USA). Rabbit anti-human Rab11a and Alexa Fluor 635 phalloidin were from Invitrogen (Carlsbad, CA, USA). Cy2-conjugated donkey anti-rabbit IgG, Cy2-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratory, (West Grove, PA, USA). All the other reagents were of analytical grade.

Animals and Experimental Protocols

Female Wistar rats (200–250 g) were used throughout. The rats had free access to food and water, and were maintained on 12:12-h automatically timed light/dark cycles. All procedures involving animals were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The rats were anesthetized with ketamine/xylazine (100 mg/3 mg/kg of b.w., i.p.), and thus maintained throughout. Body temperature was monitored with a rectal probe, and maintained at 37 °C with a heating lamp.

PRL Studies

Livers from bile-duct-cannulated rats (PE-10 tubing, Intramedic, Clay Adams) were perfused in situ, as described elsewhere [20]. TC (2 μmol/l) and CDNB (0.5 μmol/l) were added to the perfusion medium. After a 20-min equilibration period, Glu (0.1 μM) or Sal (1 μM) were added to the reservoir. Five minutes later, a 5-min basal bile sample was collected, followed by administration of E17G (3 μmol/liver, intraportal single injection over a 1-min period), or its solvent (DMSO/10 % BSA in saline [1:24]). Bile was then collected at 5-min intervals for a further 60-min period. In some experiments, a single, i.v. dose of colchicine or its inactive structural isomer lumicolchicine (1.25 mM in DMSO-saline, 1:4; 1 μmol/kg) was administered to rats via the femoral vein, 100 min before E17G administration (approximately 65 min before starting the perfusion procedure). Experiments were considered valid only if initial bile flow was greater than 30 μl/min/kg of b.w. Bile flow was determined gravimetrically, assuming a bile density of 1 g/ml. Viability of the liver was monitored by the release of lactate dehydrogenase in the perfusate outflow; experiments exhibiting activities over 20 U/l were discarded. Transport activity of Abcc2 and Abcb11 was evaluated by measuring dinitrophenyl-glutathione (DNP-G, intrahepatic metabolite of CDNB) and TC biliary excretion, respectively. Total DNP-G content was measured in bile by HPLC [21]. TC was determined by the 3α-hydroxysteroid dehydrogenase procedure [22]. Substrate outputs were calculated as the product between bile flow and substrate concentration.

For canalicular transporter localization studies, in a new set of experiments, a liver lobe was excised 20 min after addition of E17G, frozen immediately in isopentane precooled in liquid nitrogen, and stored at −70 °C for further immunofluorescence and confocal microscopy analysis. Liver sections were obtained with a Zeiss Microm HM500 microtome cryostat, air-dried, fixed with 3 % paraformaldehyde in phosphate-buffered saline and permeabilized with PBS–Triton X-100 in 2 %—BSA. After fixation, liver slices were incubated with the specific antibodies (1:100, 12 h) to Abcb11 or Abcc2, followed by incubation with the appropriate Cy2-conjugated (1:200, 1 h). Rab11a was detected by using two different specific antibodies, namely a mouse monoclonal to Rab11a antibody (1:100, 12 h) for colabeling with Abcb11 and a rabbit polyclonal to Rab11a antibody (1:100, 12 h) for colabeling with Abcc2, followed by incubation with a Cy3-conjugated donkey anti-mouse IgG and a Cy3-conjugated goat anti-rabbit IgG (1:200, 1 h), respectively. To delimit the canaliculi, F-actin was stained with Alexa Fluor 635 phalloidin (1:100, 1 h). All images were taken with a Nikon C1 Plus confocal laser scanning microscope. To ensure comparable staining and image capture performance for the different groups belonging to the same experimental protocol, liver slices were prepared on the same day, mounted on the same glass slide, and subjected to the staining procedure and confocal microscopy analysis simultaneously. Image analysis of the degree of Abcb11 and Abcc2 endocytic internalization was performed on confocal images with ImageJ 1.44p [4].

Ala Administration Studies

Fasting and alanine administration increase the pancreatic release of Glu [23–26]. The experiment was planned to potentiate the difference in Glu levels between treated and

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control rats. For this purpose, we compared the effect of alanine in 12-h-fasted rats versus control, fed animals which had free access to food the night before the experiment. In animals from both groups, the femoral vein and the common bile duct were cannulated with a polyethylene tube (PE-50 and PE-10, respectively). Saline was administered i.v. throughout the experiment to replenish body fluids. Colchicine or lumicolchicine (1.25 mM in DMSO-saline, 1:4; 1 μmol/kg) was administered via the femoral vein, 50 min before bile duct cannulation. After a 30-min equilibration period, previously fasted animals received Ala (0.5 g/kg of b.w., i.p.), whereas control, fed animals received saline. Ten min later, a 10-min basal bile sample was collected, followed by administration of E17G (15 μmol/kg of b.w. in DMSO-10 % BSA in saline, 1:24), or solvent in controls, via the femoral vein. Transport activity of Abcc2 and Abcb11 was evaluated by measuring bilirubin and total bile salt biliary excretion, respectively. Bilirubin was measured in bile by the method of Jendrassik [27]. Bile salt concentration was determined by the 3α-hydroxysteroid dehydrogenase procedure [22]. Blood samples were obtained from the tail vein both before and after 10 min of Ala administration, and glycemia was measured (Wiener Lab, Rosario, Argentina).

cAMP Measurement

In Glu and Sal administration studies, rats were anesthetized, and the portal vein was exposed. Fifteen min later, one liver lobe was excised (basal value) and rats received either Glu (0.2 μmol/kg of b.w.), Sal (4 μmol/kg of b.w.) or solvent through the portal vein. After another 10-min period, the remnant liver was excised, and 1-mg samples were homogenized in ethanol. Preparations were centrifuged (9,000 rpm, 15 min), and the supernatant was separated. Ethanol in the supernatant was evaporated, and the residue was resuspended for cAMP determination. cAMP detection was carried out by competition of [^{3}H]cAMP for carbon-dextran-immobilized PKA, as previously described [28, 29]. The pellet was resuspended in PBS with protease inhibitors, and protein was assessed in this fraction by the Lowry method [30]. Results were expressed as pmol cAMP/mg liver prot. In Ala administration studies, the amino acid was administered i.p. to fasted rats and, 10 min later, livers were excised, and a sample was processed, as described above.

Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SE). Statistical analysis was performed using one-way ANOVA, followed by the Newman–Keuls' test. The variances of the densitometric profiles of Abcb11 and Abcc2 localization were compared with the Mann–Whitney *U* test. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of the cAMP Agonists Glu and Sal on PRLs Exposed to E17G

Hepatic cAMP Levels

cAMP significantly increased 10 min after administration of either glucagon (154 ± 4 % of basal values) or salbutamol (151 ± 6 % of basal values), whereas solvent administration did not modify this parameter (97 ± 3 % of basal values). Basal hepatic cAMP was 2.2 ± 0.2 pmol/mg prot. These results confirm that 10 min are enough to raise cAMP, and this is why Glu and Sal were administered 10 min before E17G administration.

Bile Flow

E17G reduced bile flow to a nadir of 40 % of its basal value 10 min post-injection. Then, bile flow remained between 40 and 50 % of basal values over the rest of the collection period (Fig. 1 upper panels). Glu pre-administration partially prevented the fall in bile flow, this protection being independent of microtubules integrity, since colchicine treatment did not affect Glu protection (Fig. 1, upper right panel). Sal also protected from E17G-induced cholestasis. However, the adrenergic agonist did not prevent the E17G maximal cholestatic effect but rather accelerated bile flow recovery following this nadir. Sal effects were completely abolished by colchicine, thus confirming the role for microtubules in its protective effect.

Abcc2 and Abcb11 Transport Activities

E17G reduced the biliary excretion of DNP-G and TC to a minimum of approximately 35 % of their basal values, 10 min post-injection. Both transport activities recovered to approximately 60 % of basal values from 20 min after E17G administration onwards (Fig. 1, middle and lower panels). This recovery was independent of microtubules, since colchicine did not affect this process. Glu prevented partially, but significantly, the decrease in the excretion of both substrates induced by E17G 10 min post-injection. After that, livers treated with Glu reached similar excretion rates as the control ones. The global excretions of DNP-G and TC in the group receiving Glu and E17G were more similar to controls than to the group receiving E17G alone. Initially, Glu protection was independent of microtubules, but in livers treated with colchicine, Glu effect on TC

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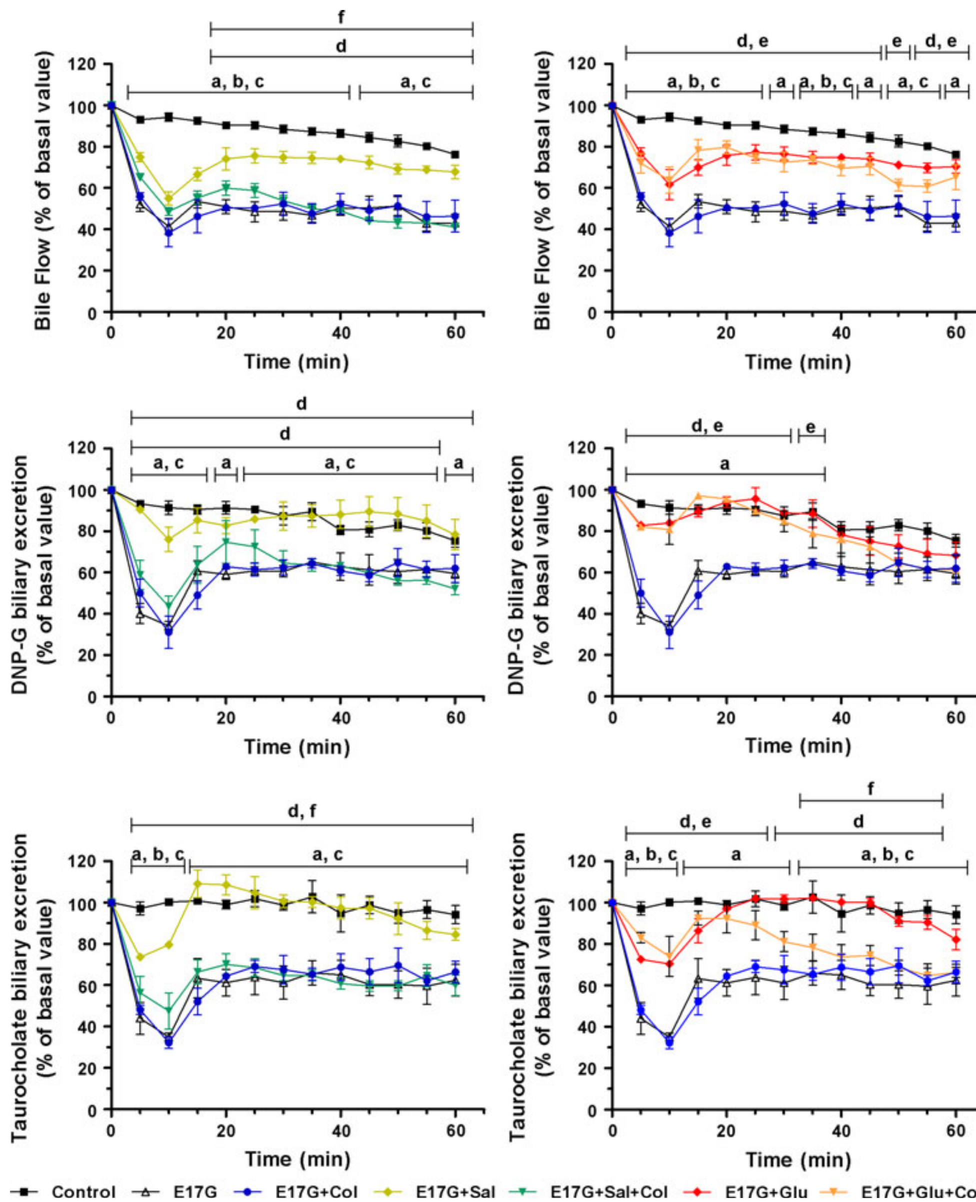

Fig. 1 Glucagon (*Glu*) and salbutamol (*Sal*) protect against E17G-induced impairment of bile flow and biliary secretion of dinitrophenyl-glutathione (*DNP-G*) and taurocholate (*TC*) in the perfused rat liver (*PRL*). Temporal changes in bile flow (*upper panel*) and in the biliary excretion rate of both DNP-G (*middle panel*) and TC (*lower panel*) throughout the perfusion period. PRLs were treated with a bolus of E17G (3 µmol/liver), or with the E17G vehicle (DMSO/BSA 10 % in saline) in controls, in the presence or absence of Glu (0.1 µM, *right panels*) or Sal (1 µM, *left panels*). The effect of Sal and Glu on E17G cholestatic effect in PRLs from rats pretreated with the microtubule-disrupting agent colchicine (Col, 1 µmol/kg, 100 min before E17G administration) is also shown. The effects of Glu or Sal alone on the parameters studied did not differ from those of the control group (not depicted for the sake of clarity). [a]Control significantly different from E17G ($p < 0.05$), [b]Control significantly different from E17G + Sal or E17G + Glu ($p < 0.05$), [c]Control significantly different from E17G + Sas + Col or E17G + Glu + Col ($p < 0.05$), [d]E17G significantly different from E17G + Sal or E17G + Glu ($p < 0.05$), [e]E17G significantly different from E17G + Sal + Col or E17G + Glu + Col ($p < 0.05$), and [f]E17G + Sal or E17G + Glu significantly different form E17G + Sal + Col or E17G + Glu + Col ($p < 0.05$). E17G + Col results were included in the ANOVA tests, but comparisons with this group were not shown in the figure for the sake of clarity. $n = 3$–6 animals per group

excretion tended to disappear from min 25 onwards (Fig. 1, lower right panel), this effect being completely abolished from min 35 onwards. This is reflected also as a significant decrease in the total excretion of TC over the 60-min collection period (Table 1). Glu protection of DNP-G excretion was not affected by colchicine throughout-(Fig. 1, middle right panel, and Table 1). Like Glu, Sal prevented E17G impairment of both TC and DNP-G

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Table 1 Impairment of TC and DNP-G outputs by E17G in the perfused rat liver and their prevention by salbutamol and glucagon

	TC	DNP-G
Control	98 ± 2 %	85 ± 2 %
Glu	100 ± 4 %	88 ± 5 %
Sal	98 ± 4 %	86 ± 4 %
E17G	59 ± 6 %[a]	59 ± 3 %[a]
E17G + Col	61 ± 3 %[a]	58 ± 2 %[a]
E17G + Glu	91 ± 1 %[b]	82 ± 3 %[b]
E17G + Glu + Col	78 ± 4 %[a,b,c]	79 ± 5 %[b]
E17G + Sal	95 ± 3 %[b]	85 ± 6 %[b]
E17G + Sal + Col	62 ± 4 %[a,d]	62 ± 4 %[a,d]

Results are expressed as the average cumulative biliary excretion of each substrate over the 60-min period of bile collection, compared to basal excretion (expressed as % of basal values)

E17G, estradiol-17β-D-glucuronide; *Sal* salbutamol; Glu, glucagon; Col, colchicine

Taurocholate (TC) and dinitrophenyl-glutathione (DNP-G) outputs were measured in bile collected for 60 min in 5-min periods after E17G administration, as depicted in Fig. 1. Basal bile was collected for 10 min, immediately before E17G injection. Glu (0.1 μM) and Sal (1 μM) were administered to the reservoir 10 min before addition of E17G (3 μmol/liver) to the perfusion media. Control livers received only the vehicle (saline). Animals from Col group received Col (1 μmol/kg) 100 min before E17G (60 min before surgery). Ancillary experiments administrating the inactive colchicine analogue lumicolchicine instead of colchicine to control, E17G, E17G + Glu, and E17G + Sal groups revealed that lumicolchicine had no independent effect on the parameters studied (data not shown)

Results are expressed as mean ± SE for 3 animals per group

[a] Statistically different from control group ($p < 0.05$)

[b] Statistically different from E17G group ($p < 0.05$)

[c] Statistically different from E17G + Glu group ($p < 0.05$)

[d] Statistically different from E17G + Sal group ($p < 0.05$)

excretion. However, as for bile flow, this protection was fully dependent on microtubule integrity (Fig. 1, middle and lower left panels, and Table 1). Livers treated with Glu or Sal alone did not differ from control livers, either in bile flow or in the biliary excretion of DNP-G and TC (data not shown). This indicates that none of these compounds affect the activity of canalicular or basolateral transporters involved in TC and DNP-G transport, at least at the concentration used here.

Abcc2 and Abcb11 Localization

Confocal images showed that, in E17G-treated livers, both Abcb11 (Fig. 2a) and Abcc2 (Fig. 3a) were detected in intracellular vesicles, consistent with their endocytic internalization from the canalicular membrane. As previously described using the isolated rat hepatocyte couplet model [19], part of this transporter-associated fluorescence colocalized with Rab11a (Figs. 2 and 3, arrowheads in amplified images). Sal treatment led all transporters back to the canalicular domain. In turn, with Glu, part of these transporter-containing vesicles internalized by the estrogen remained colocalized with Rab11a in an inner region (Figs. 2 and 3, arrowheads in amplified images). This pattern of internalization was evident in some canalicular structures, and coexisted with preserved localization of the transporters in other canaliculi. In accordance with confocal images, densitometric studies showed that E17G-treated livers exhibit a wider and flatter profile, consistent with increased transporter-associated fluorescence at a greater distance from the canalicular membrane, indicative of internalization of Abcb11 (Fig. 2b, left panel) and Abcc2 (Fig. 3b, right panel). In livers perfused with either E17G + Sal or E17G + Glu, the distribution of both Abcb11 and Abcc2 was almost identical to that of control livers, as confirmed by densitometric analysis. The densitometric profile of actin-associated fluorescence used to delineate the canaliculi showed that the treatments did not alter the canalicular width (Figs. 2b, 3b, right panels).

Effect of Ala Pretreatment In Vivo

Hepatic cAMP Levels

Ten min after alanine administration, cAMP in livers from fasted rats increased from 1.9 ± 1 pmol/mg prot. to 4.3 ± 0.4 pmol/mg prot., which was significantly higher than the levels of cAMP in livers from control, fed rats treated with saline (1.8 ± 0.5 pmol/mg prot.). Therefore, this protocol for Ala administration was used in bile flow and biliary secretion studies.

Plasma Glucose Levels

Glycemia was not modified 10 min after treatment with Ala in fasted rats (basal: 73 ± 3 mg/dl; 10 min: 83 ± 4 mg/dl). As expected, these values were lower than those in fed rats (basal: 106 ± 4 mg/dl; 10 min after saline administration: 105 ± 7 mg/dl). Notwithstanding the differences, all values are within the normal range of glycemia.

Bile Flow

In vivo, E17G reduced bile flow in fed rats to a minimum of 27 % of basal bile flow, at 20 min post-injection (Fig. 4, upper panel). Ala pretreatment did not prevent the maximum decay in bile flow, but accelerated the recovery of this parameter to 70 % of its basal value. This protection was independent of microtubules during the first 60 min but, thereafter, bile flow decreased in the group of rats pretreated with colchicine, eventually reaching bile flow

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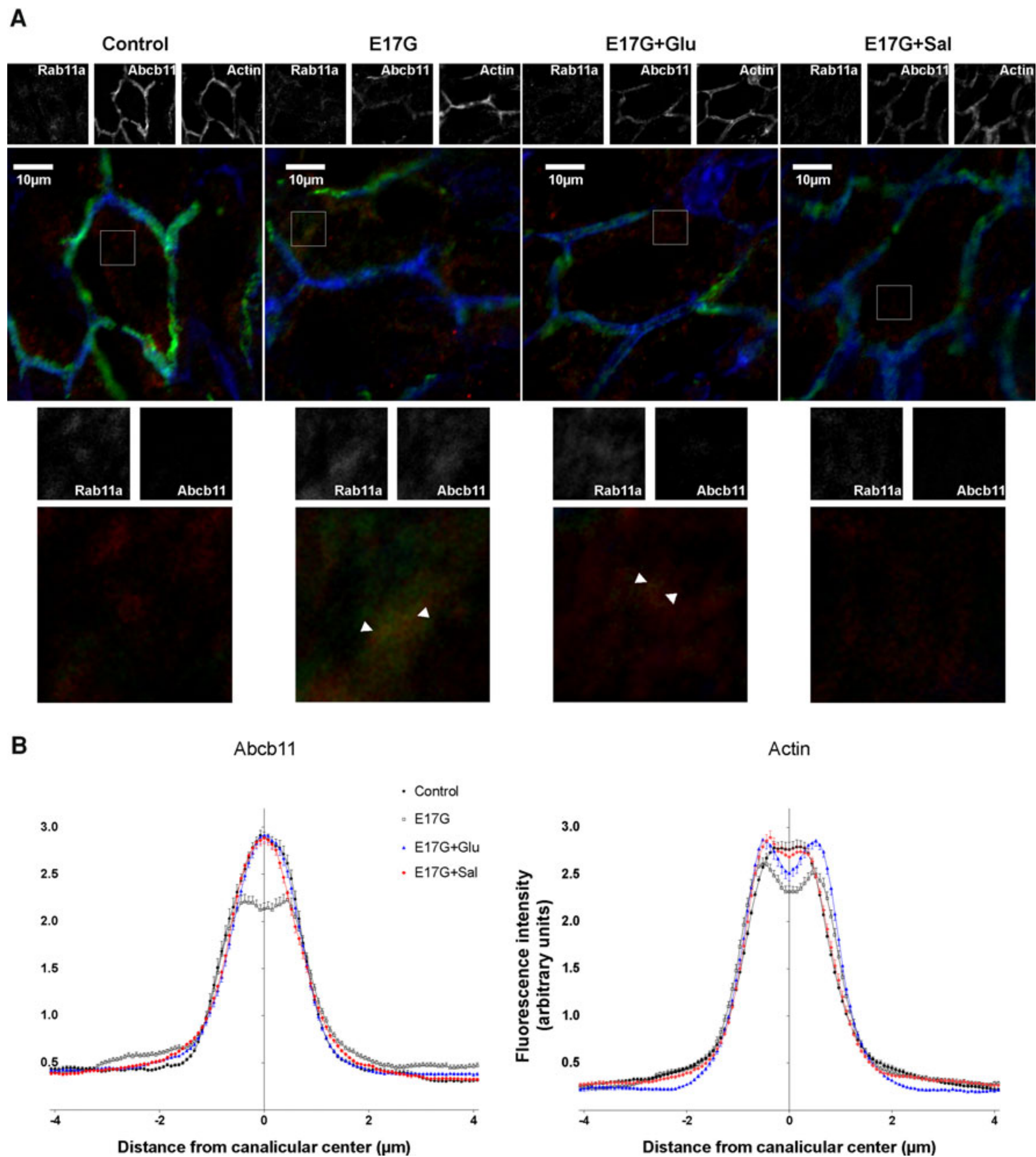

Fig. 2 Glucagon (*Glu*) and salbutamol (*Sal*) protect against E17G-induced impairment in Abcb11 localization in the perfused rat liver (*PRL*). **a** Representative confocal images of immunostained liver samples displaying the canalicular transporters Abcb11 (*green*), the endosomal marker Rab11a (*red*), actin (*blue*), and merged images (*yellow*). Square-demarcated zones are shown amplified below the respective microphotographs. In control livers, Abcb11 was mainly confined to the canalicular space, as delineated by actin staining. E17G induced a clear internalization of transporter-containing vesicles outside of the canalicular region, which partially colocalized with Rab11a (*arrowheads*); this phenomenon was significantly prevented by both Glu and Sal. The E17G + Glu group shows some transporter-containing vesicles that remained mainly colocalized with the endosomal marker Rab11a, in a deep intracellular compartment (*arrowheads*). **b** Densitometric analysis of fluorescence intensity profile of Abcb11 (*left images*) and actin (*right images*). Graphs depict the intensity of fluorescence associated with the transporters along an 8-μm line (from −4 μm to +4 μm of the canalicular center), perpendicular to the canaliculus. In control livers, transporter-associated fluorescence was concentrated in the canalicular space. E17G-induced retrieval of transporters from the canalicular membrane was detected as a decrease in the fluorescence intensity in the canalicular area, together with an increased fluorescence at a greater distance from the canaliculus ($p < 0.01$ vs. control). Distribution profiles of livers treated with E17G + Glu and E17G + Sal were similar to controls, thus indicating a significant decrease of Abcb11 internalization ($p < 0.01$ vs. E17G). ($n = 20$–50 canaliculi per preparation, from three independent preparations). Statistical analysis of the distribution profiles of actin, used to demarcate the limits of the canaliculi, showed that none of the treatments induced changes in the normal distribution of this cytoskeletal protein

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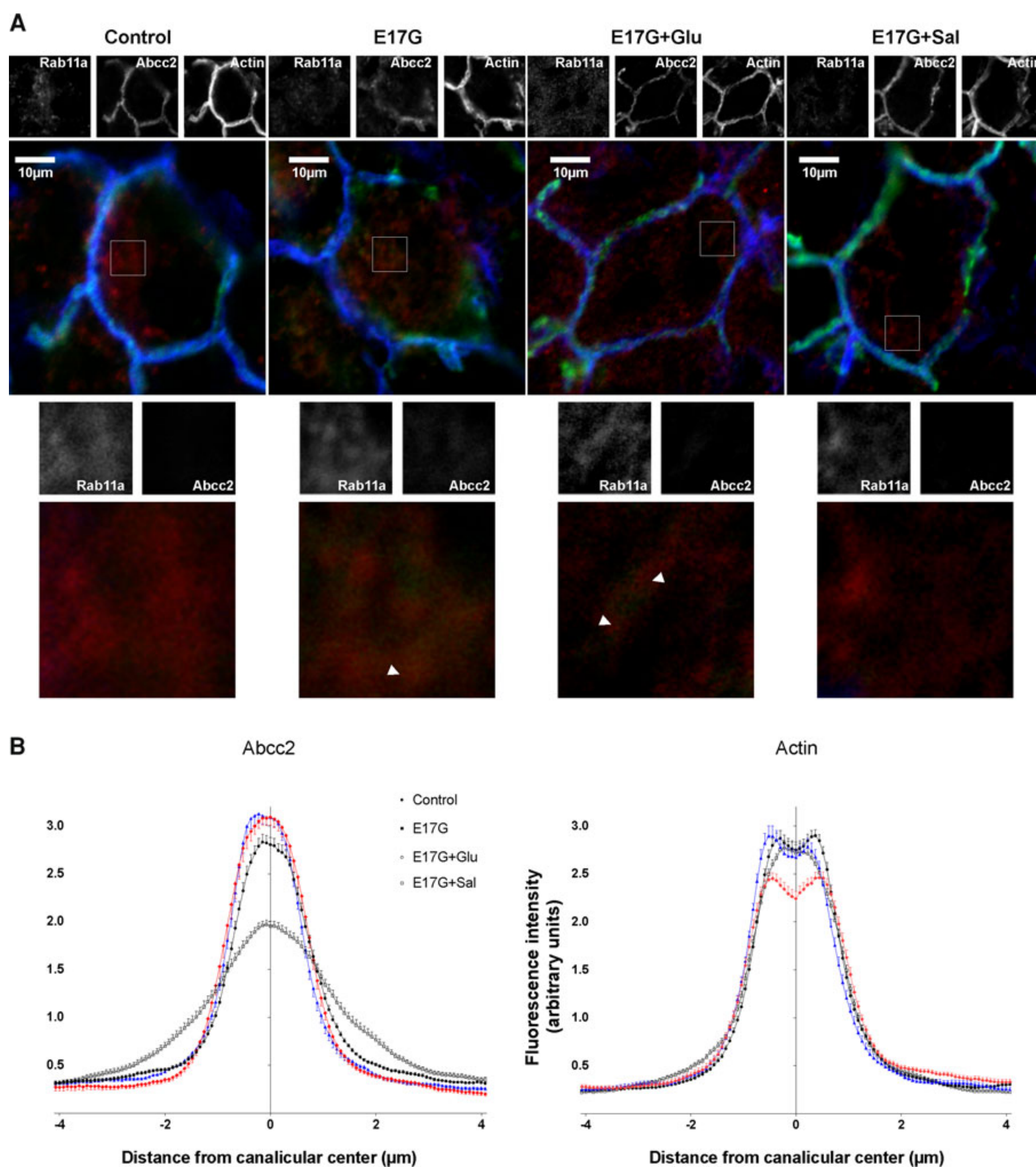

Fig. 3 Glucagon (Glu) and salbutamol (Sal) protect against E17G-induced impairment in Abcc2 localization in the perfused rat liver (PRL). **a** Representative confocal images of immunostained liver samples displaying the canalicular transporter Abcc2 (*green*), the endosomal marker Rab11a (*red*), actin (*blue*), and merged images (*yellow*) of PRLs subjected to the different treatments. Square-demarcated zones are shown amplified below the respective micro-photographs. In control livers, Abcc2 was mainly confined to the canalicular space, as delineated by actin staining. E17G induced a clear internalization of transporter-containing vesicles outside of the canalicular region, which partially colocalized with the endosomal marker Rab11a (*arrowheads*); this phenomenon was significantly prevented by Glu and Sal. Image from the E17G + Glu group shows some transporter-containing vesicles that remained mainly colocalized with Rab11a in a deep, intracellular compartment (*arrowheads*). **b** Densitometric analysis of fluorescence intensity profile of Abcc2 (*left images*) and actin (*right images*). Graphs depict the intensity of fluorescence associated with the transporters along an 8-μm line (from −4 μm to +4 μm of the canalicular center), perpendicular to the canaliculus. In control livers, transporter-associated fluorescence was concentrated within the canalicular space. E17G-induced retrieval of transporters from the canalicular membrane was detected as a decrease in the fluorescence intensity in the canalicular area, together with an increased fluorescence at a greater distance from the canaliculus ($p < 0.01$ vs. controls). Distribution profiles of livers treated with E17G + Glu and E17G + Sal were similar to control, thus indicating a significant decrease of Abcc2 internalization ($p < 0.01$ vs. E17G, $n = 20$–50 canaliculi per preparation, from three independent preparations). Statistical analysis of the distribution profiles of actin, used to demarcate the limits of the canaliculi, showed that none of the treatments induced changes in the normal distribution of this cytoskeletal protein

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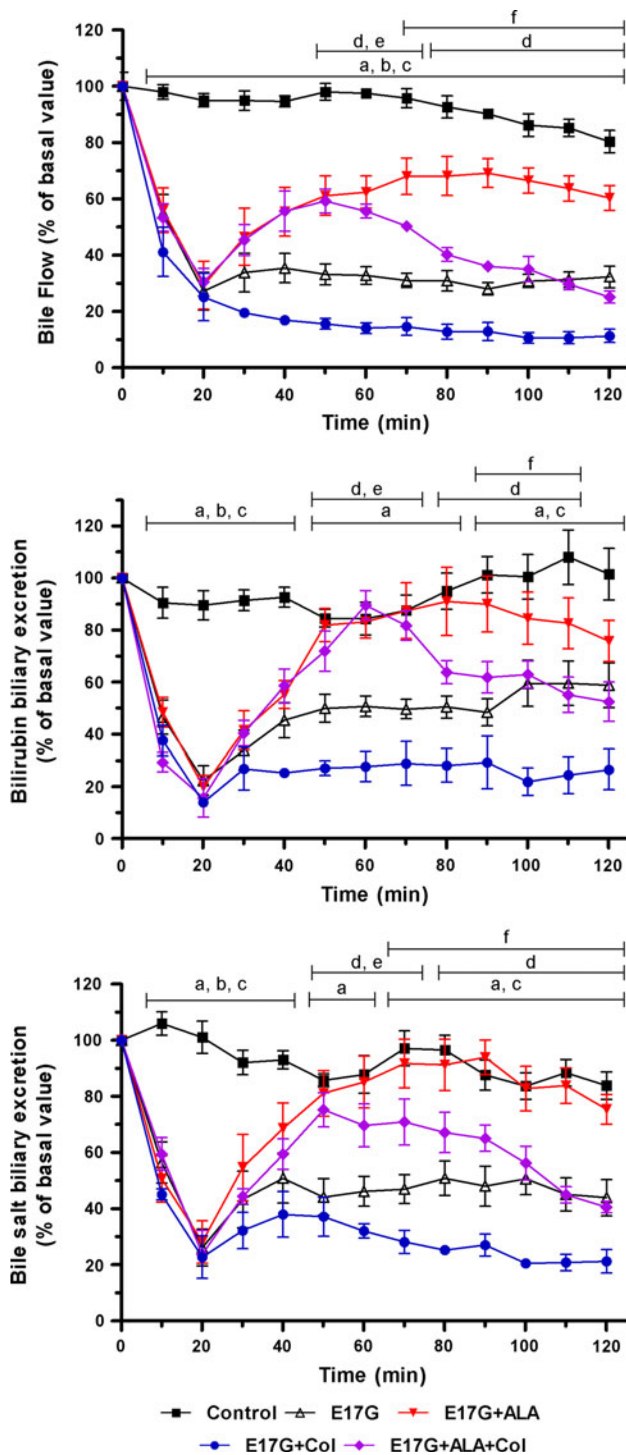

Fig. 4 Intraperitoneal administration of alanine (*Ala*) protects against E17G-induced impairment of bile flow and biliary secretion of total bilirubin (*BR*) and total bile salts (*BSs*) in the whole rat. Temporal changes in bile flow (*upper panel*) and in the biliary excretion rate of both BR (*middle panel*) and BSs (*lower panel*) after the injection of E17G (3 µmol/liver, i.v.), or the E17G vehicle DMSO in controls. Rats from Ala group were fasted overnight, and received an injection of alanine (0.5 g/kg of b.w., i.p.), 10 min before E17G administration. Control rats had free access to food the night before the experiment. The effect of Ala administration on the cholestatic effect E17G in vivo in rats pretreated with the microtubule-disrupting agent colchicine (1 µmol/kg of b.w., 100 min before E17G administration) is also shown. Ala alone had no independent effect on the parameters studied; these results were not depicted in the figure for the sake of clarity. [a]Control significantly different from E17G ($p < 0.05$), [b]Control significantly different from E17G + ALA ($p < 0.05$), [c]Control significantly different from E17G + ALA + Col ($p < 0.05$), [d]E17G significantly different from E17G + ALA ($p < 0.05$), [e]E17G significantly different from E17G + ALA + Col ($p < 0.05$), [f]E17G + ALA significantly different form E17G + ALA + Col ($p < 0.05$). E17G + Col results were included in the ANOVA test, but comparisons with this group were not shown in the figure for the sake of clarity. $n = 4$–8 animals per group

values similar to E17G-treated rats; this suggests the need for microtubules to sustain the recovery of bile flow with time.

Abcc2 and Abcb11 Transport Activities

E17G reduced the excretion of both bilirubin and bile salts to a minimum of approximately 25 % of basal values, followed afterwards by a partial recovery of these parameters (Fig. 4, middle and lower panels). Ala did not protect rats from the initial fall in the excretions of bilirubin and bile salts, but accelerated their recovery to control values soon after the initial decay. Overall, Ala pretreatment restored half of the excretion rate fall induced by E17G (Table 2). Similarly to what happened to bile flow, the early recovery soon after the initial decay was not affected by colchicine. However, later on (from 50 to 60 min onwards), biliary excretion of bile salts and bilirubin decreased so as to become different from E17G + Ala group from 70 and 90 min, respectively, and reaching values similar to those of the E17G-treated group at the end of the perfusion period.

Discussion

E17G administration to rats produces a decrease in bile flow and in the biliary excretion of Abcb11 [4] and Abcc2 [10, 11] substrates. This decrease in transport activity may be causally attributed to internalization of transporters from the canalicular membrane into cytoplasmic vesicles belonging to the subapical endosomal compartment. There is a constitutive recycling of ABC transporters from the canalicular membrane into this subapical compartment, and vice versa [31]. E17G breaks the equilibrium by favoring transporter internalization. Hence, this alteration could be theoretically prevented, or even reversed, by substances that favor the reinsertion of transporters, so as to restore the equilibrium. One compound bearing this property is cAMP. Roelofsen et al. [14] showed that cAMP participates in the three steps of reinsertion of Abcc2

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Table 2 Impairment of bile salt and bilirubin output by E17G in vivo, and its prevention by alanine

	Bile salt	BR
Control	92 ± 6 %	94 ± 4 %
Ala	92 ± 1 %	94 ± 7 %
E17G	46 ± 5 %[a]	48 ± 4 %[a]
E17G + Ala	74 ± 4 %[a,b]	70 ± 5 %[a,b]
E17G + Col	20 ± 2 %[a,b]	19 ± 3 %[a,b]
E17G + Ala + Col	57 ± 3 %[a,c]	57 ± 4 %[a,c]

Results are expressed as the average cumulative biliary excretion of each substrate over 2 h of bile collection, compared to basal excretion (expressed as % of basal values)

E17G, estradiol-17β-D-glucuronide; Ala, alanine; Col, colchicine

Bile salt (BS) and bilirubin (BR) output were measured in bile collected for 2 h. in 10-min periods after E17G administration, as depicted in Fig. 4. Basal bile was collected for 10 min, immediately before E17G injection. Ala (0.5 g/kg of b.w., i.p.) was administered 10 min before E17G (15 μmol/kg of b.w., i.v.) in fasted animals, whereas E17G alone was administered to fed animals (see materials and methods). Control group was fed, and received vehicle administration. Ala group was fasted, and received Ala. Ancillary experiments administrating the inactive colchicine analogue lumicolchicine instead of colchicine to control, E17G, and E17G + Ala groups revealed that lumicolchicine had no independent effect on the parameters studied (data not shown). Results are expressed as mean ± SE, for 3–8 animals per group

[a] Statistically different from control group ($p < 0.05$)

[b] Statistically different from E17G group ($p < 0.05$)

[c] Statistically different from E17G + Ala group ($p < 0.05$)

following the initial redistribution of the transporter occurring after isolation of rat hepatocyte couplets i.e. (1) endocytosis from the basolateral plasma membrane where Abcc2 is initially redistributed, (2) its transcytosis to the apical pole in a microtubule-dependent manner, and (3) the fusion of transporter-containing vesicles with the apical membrane, in a microtubule-independent manner. In agreement with this, cAMP prevents canalicular transporter retrieval in cholestasis [4, 10] by stimulating the apically directed vesicular pathway.

Recently, we demonstrated in isolated rat hepatocyte couplets that two hormones that increase intrahepatic cAMP, Glu and adrenaline (the latter via β2 receptor), both prevent E17G-induced impairment of Abcb11 and Abcc2 transport [19]. Surprisingly, the underlying cAMP-mediated mechanisms of protection afforded by both hormones differ with each other. Glu affords prevention via a PKA-dependent, microtubule-independent mechanism, whereas Sal (a β2 agonist) does so via an Epac-MEK-mediated, microtubule dependent mechanism. In that work, we had also showed confocal images demonstrating that the pools of transporter-containing vesicles whose reinsertions were stimulated by these two hormonal pathways were different. Indeed, E17G induces internalization of transporters into vesicles that colocalize with Rab11a, an endosomal marker involved in the normal recycling of these transporter-containing vesicles. Sal fully reinserts transporters whereas Glu leaves some transporters colocalized with the endosomal marker in a deeper, endosomal compartment. Overall, this strongly suggests the existence of two different pools of transporter-containing vesicles; glucagon would reinsert transporters present in a vesicular compartment underneath of the canalicular membrane via a PKA-dependent, microtubule-independent mechanism, whereas Sal does so by mobilizing transporter-containing vesicles from a deeper compartment to the membrane with the aid of microtubules, via an Epac/MEK-dependent mechanism.

Here, we ascertained whether these cAMP-elevating hormones are also able to prevent the impairment in bile flow induced by E17G in a more physiological model, such as the perfused rat liver; in this model, it is possible to evaluate the time course of the prevention, and it is therefore useful to determine whether these compounds exert their beneficial effect at the initiation of cholestatic event or during the recovery phase that follows the initial insult. This discrimination is relevant, since these two processes involve different set of signaling mediators and differential requirement for cytoskeleton structures.

Glu and Sal increased liver cAMP in a similar magnitude. Both compounds also prevented the alterations in bile flow and Abcc2 and Abcb11 transport activities induced by the estrogen. Glu was able to prevent the maximal decrease in bile flow produced by E17G administration; this differs from Sal in that, although the latter showed a clear trend towards prevention, it did not reach statistical significance. However, the differential behavior of Glu and Sal did not apply for both DNP-G and TC biliary excretion, since both agonists prevented the maximal effect of E17G. This strongly suggests that Sal, like Glu, is able to prevent the early events triggering estrogen-induced cholestasis. Similarly to what had been observed in hepatocyte couplets [19], Glu and Sal differed in their mechanisms of protection. During the period of bile collection, Sal prevention fully depended on microtubule integrity, whereas Glu prevention was only belatedly affected by colchicine. Confocal images showed that Sal fully prevented the internalization of both Abcb11 and Abcc2 induced by E17G, whereas Glu only partially prevented estrogen-induced transporter internalization, since some transporter-containing vesicles remained colocalized with Rab11a at inner regions of the cell. Intracellular colocalization of canalicular transporters with Rab11a occurs during their normal recycling between the canalicular membrane and the subapical compartment, or under an endocytic stimulus [32, 33]. According to these findings, two different intracellular pools of transporters would exist. There is a pool of vesicles containing

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transporters that had been internalized by E17G into a deep compartment, from where a long-distance, microtubule-dependent trafficking is needed for reinsertion. On the other hand, there is another pool composed of vesicles that are near the canalicular membrane, ready to be reinserted by Glu using a microtubule-independent mechanism, as described in vitro in isolated rat hepatocyte couplets [19]. On this base, a possible explanation for the late dependency of Glu on microtubules may be hypothesized. The initial prevention exerted by Glu would depend on the rapid reinsertion of a pool of transporter-containing vesicles that is localized near the canalicular membrane, a process that would not require microtubules. With time, this subapical pool would become exhausted, and needs to be replenished either by newly synthesized transporters or by transporters delivered from a deeper endosomal compartment. In the presence of colchicine, the long-distance trafficking of these transporters is blocked, and the pool of readily available transporters underneath of the canalicular membrane would become exhausted. It is highly probable that those transporter-containing vesicles whose trafficking is not stimulated by Glu and that remained colocalized with Rab11a (as observed in confocal images) are implied in the replenishment of the subapical compartment via a microtubule-dependent vesicular pathway. An alternative explanation for Glu late-dependence on microtubules involves dissipation of cAMP. Inhibition of microtubule polymerization by colchicine would affect the microdomain where glucagon-derived cAMP is acting, and would thereby produce a dissipation of this intracellular messenger. However, this phenomenon is highly unlikely to account for our results. Indeed, since colchicine was administered long before Glu (about 80 min ahead), dissipation of cAMP would have affected every time point of the experiments. Therefore, the initial protection exerted by Glu could not have been observed. Regarding the microtubule dependency of Sal effects observed in our PRL experiments, it is likely that Sal effects depend on the continuous mobilization of deeper internalized vesicles, as previously concluded from our in vitro findings [19]. Hence, in PRLs, the reinsertion of transporters mediated by cAMP after E17G insult relies on pathways that are dependent and independent on microtubule integrity. This finding evidences the relevance of studies using either PRL, or animals in vivo, where the time course of the cholestasis can be evaluated, thus complementing in vitro studies where signaling pathways can be easily studied.

Stieger et al. [8] proposed an alternative explanation for the cholestatic effects of E17G. They provided evidence that E17G trans-inhibits Abcb11-mediated transport of bile salts when the estrogen is transported via Abcc2 into canalicular plasma membrane vesicles and Sf9 insect cell-derived vesicles expressing Abcb11 and Abcc2. The question is whether the prevention by Glu or Sal against E17G-induced impairment in the biliary excretion of substrates could be attributed to a decrease in the trans-inhibition of Abcb11. The effect of both agonists, Glu and Sal, is mediated by cAMP. Since cAMP stimulates the insertion of Abcc2 in the canalicular membrane [14, 19], the effects of Glu and Sal lead to an increase in the amount of Abcc2 in the canalicular membrane in the presence of the cholestatic estrogen. As a consequence, the transport of E17G into bile via Abcc2 would have been enhanced, and therefore a greater E17G-mediated trans-inhibition of Abcb11 and the resulting exacerbated cholestatic effect would have been expected. On the contrary, we observed that both Abcc2 pro-inserting compounds afforded preventive effects. Hence, the trans-inhibition phenomenon seems not to be relevant in more physiological models, like those involving PRLs or intact rats. In line with this, Huang et al. [34] presented evidence against the capability of E17G to induce cholestasis via trans-inhibition of Abcb11-mediated bile salt transport, since they showed that accumulation of E17G in bile is not sufficient to induce cholestasis in transgenic TR^{-} rats lacking Abcc2.

As a potential therapeutic agent, Glu has the disadvantage of affecting plasmatic glucose levels. In vivo, Glu pancreatic secretion is regulated by the diet, particularly by its protein content. Once released by the pancreas, Glu enters the portal circulation and reaches the liver prior to entering the systemic circulation; therefore, the liver is exposed to high levels of this hormone. Amino acid administration, particularly Ala, increases Glu levels in the portal vein, thus mimicking the effect of a protein-rich diet; moreover, Ala increases insulin levels, thus attenuating changes in glycemia [23–26]. Hence, we used this model to increase intrahepatic cAMP levels in vivo without affecting glucose levels. As expected, Ala prevented E17G-induced cholestasis in vivo. Contrary to what was observed for Glu and Sal in the PRL, Ala was not able to prevent the maximal decrease in bile flow and in both bilirubin and bile salt excretion produced by E17G. Nevertheless, Ala accelerated the recovery of these parameters after the nadir, reaching values similar to controls in the excretion of the Abcb11 and Abcc2 solutes. Although the reason for the lack of prevention of the maximal decrease in bile flow by Ala in vivo cannot be addressed from our findings, it probably depends either on the model itself or on the different protocols used to produce cholestasis in vivo and in PRLs. Glu or Sal increases cAMP by a direct action on the hepatocyte, whereas Ala needs the mediation of a hormone most likely Glu. While similar increments in hepatic cAMP were reached 10 min after administration of either Ala in vivo ($\sim$140 %) or Glu in PRLs ($\sim$155 %), the actual cAMP levels in the compartment involved in protection against the E17G insult might have increased later in vivo

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than in PRLs, thus being lower at the moment of the cholestatic insult, but higher enough during the recovery phase.

The prevention of E17G-induced cholestasis afforded by Ala administration indicates that it is possible to modulate cAMP with diets, and to reach levels high enough to prevent cholestasis. To confirm the nature of this protection, dependency on microtubules was analyzed. The microtubule disruptor colchicine did not affect the protection afforded by Ala during the first hour after E17G injection, thus indicating a microtubule-independent phenomenon similar to that observed in Glu experiments, both in PRLs and in hepatocyte couplets. After 60 min, bile flow and the biliary excretion of bilirubin and bile salts decreased to levels lower than those in E17G-treated rats. This is consistent with the late microtubule dependency of Glu protection discussed above. Because of the longer period of study in Ala in vivo experiments as compared to those of Glu in PRLs (120 vs. 60 min, respectively), the proposed requirement for a normal microtubule-dependent supply of transporter-containing vesicles from Golgi to replenish the subapical pool would become more critical in in vivo experiments.

In conclusion, Glu and Sal-mediated increase in hepatic cAMP can protect against E17G-induced cholestasis not only in vitro but also in the PRL. A similar protection can be evoked in vivo, by increasing hepatic cAMP via Ala administration. This opens the possibility of using treatments that increase hepatic cAMP levels as therapeutic agents in estrogen-induced cholestasis. One of these treatments could involve the modulation of hormones like Glu by means of, for example, a protein-rich diet.

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Conflict of interest None.

References

1. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. *Annu Rev Biochem*. 2002;71:537–592.
2. Gatmaitan ZC, Arias IM. ATP-dependent transport systems in the canalicular membrane of the hepatocyte. *Physiol Rev*. 1995;75: 261–275.
3. Esteller A. Physiology of bile secretion. *World J Gastroenterol*. 2008;14:5641–5649.
4. Crocenzi FA, Mottino AD, Cao J, et al. Estradiol-17beta-D-glucuronide induces endocytic internalization of Bsep in rats. *Am J Physiol Gastrointest Liver Physiol*. 2003;285:G449–G459.
5. Trauner M, Meier PJ, Boyer JL. Molecular regulation of hepatocellular transport systems in cholestasis. *J Hepatol*. 1999;31: 165–178.
6. Vore M, Liu Y, Huang L. Cholestatic properties and hepatic transport of steroid glucuronides. *Drug Metab Rev*. 1997;29: 183–203.
7. Adlercreutz H, Tikkanen MJ, Wichmann K, et al. Recurrent jaundice in pregnancy. IV. Quantitative determination of urinary and biliary estrogens, including studies in pruritus gravidarum. *J Clin Endocrinol Metab*. 1974;38:51–57.
8. Stieger B, Fattinger K, Madon J, et al. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology*. 2000;118: 422–430.
9. Vallejo M, Briz O, Serrano MA, et al. Potential role of trans-inhibition of the bile salt export pump by progesterone metabolites in the etiopathogenesis of intrahepatic cholestasis of pregnancy. *J Hepatol*. 2006;44:1150–1157.
10. Mottino AD, Cao J, Veggi LM, et al. Altered localization and activity of canalicular Mrp2 in estradiol-17beta-D-glucuronide-induced cholestasis. *Hepatology*. 2002;35:1409–1419.
11. Mottino AD, Crocenzi FA, Pozzi EJ, et al. Role of microtubules in estradiol-17beta-D-glucuronide-induced alteration of canalicular Mrp2 localization and activity. *Am J Physiol Gastrointest Liver Physiol*. 2005;288:G327–G336.
12. Roma MG, Crocenzi FA. Sanchez Pozzi EA. Hepatocellular transport in acquired cholestasis: new insights into functional, regulatory and therapeutic aspects. *Clin Sci (Lond)*. 2008;114: 567–588.
13. Kipp H, Arias IM. Trafficking of canalicular ABC transporters in hepatocytes. *Annu Rev Physiol*. 2002;64:595–608.
14. Roelofsen H, Soroka CJ, Keppler D, et al. Cyclic AMP stimulates sorting of the canalicular organic anion transporter (Mrp2/cMoat) to the apical domain in hepatocyte couplets. *J Cell Sci*. 1998;111: 1137–1145.
15. Steinberg SF, Chow YK, Bilezikian JP. Cyclic AMP and guanine nucleotide regulatory proteins. In: Arias IM, Jakoby WB, Popper H, eds. *The Liver: Biology and Pathobiology*. 2nd ed. New York: Raven Press, Ltd; 2003:769–776.
16. Branum GD, Bowers BA, Watters CR, et al. Biliary response to glucagon in humans. *Ann Surg*. 1991;213:335–340.
17. Morgan NG, Blackmore PF, Exton JH. Age-related changes in the control of hepatic cyclic AMP levels by alpha 1- and beta 2-adrenergic receptors in male rats. *J Biol Chem*. 1983;258: 5103–5109.
18. Bollen M, Keppens S, Stalmans W. Specific features of glycogen metabolism in the liver. *Biochem J*. 1998;336:19–31.
19. Zucchetti AE, Barosso IR, Boaglio A, et al. Prevention of estradiol 17beta-D-glucuronide-induced canalicular transporter internalization by hormonal modulation of cAMP in rat hepatocytes. *Mol Biol Cell*. 2011;22:3902–3915.
20. Crocenzi FA. Sanchez Pozzi EJ, Ruiz ML et al. Ca(2 +)-dependent protein kinase C isoforms are critical to estradiol 17beta-D-glucuronide-induced cholestasis in the rat. *Hepatology*. 2008;48:1885–1895.
21. Hinchman CA, Matsumoto H, Simmons TW, et al. Intrahepatic conversion of a glutathione conjugate to its mercapturic acid. Metabolism of 1-chloro-2,4-dinitrobenzene in isolated perfused rat and guinea pig livers. *J Biol Chem*. 1991;266:22179–22185.
22. Talalay P. Enzymic analysis of steroid hormones. *Methods Biochem Anal*. 1960;8:119–143.
23. Muller WA, Faloona GR, Unger RH. The effect of alanine on glucagon secretion. *J Clin Invest*. 1971;50:2215–2218.
24. Porcellati F, Pampanelli S, Rossetti P, et al. Effect of the amino acid alanine on glucagon secretion in non-diabetic and type 1 diabetic subjects during hyperinsulinaemic euglycaemia, hypoglycaemia

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and post-hypoglycaemic hyperglycaemia. *Diabetologia*. 2007;50: 422–430.

25. Rocha DM, Faloona GR, Unger RH. Glucagon-stimulating activity of 20 amino acids in dogs. *J Clin Invest*. 1972;51: 2346–2351.
26. Tanaka K, Inoue S, Saito S, et al. Hepatic vagal amino acid sensors modulate amino acid-induced insulin and glucagon secretion in the rat. *J Auton Nerv Syst*. 1993;42:225–231.
27. Jendrassik J, Grof P. Vereinfachte photometrische Methode zur Bestimmung des Blut-bilirubins. *Biochem Z*. 1938;297:81–89.
28. Davio CA, Cricco GP, Bergoc RM, et al. H1 and H2 histamine receptors in *N*-nitroso-*N*-methylurea (NMU)-induced carcinomas with atypical coupling to signal transducers. *Biochem Pharmacol*. 1995;50:91–96.
29. Sabbatini ME, Villagra A, Davio CA, et al. Atrial natriuretic factor stimulates exocrine pancreatic secretion in the rat through NPR-C receptors. *Am J Physiol Gastrointest Liver Physiol*. 2003; 285:G929–G937.
30. Lowry OH, Rosembrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193: 265–275.
31. Wakabayashi Y, Arias IM. Apical recycling of canalicular ABC transporters. In: Arias IM, Wolkoff A, Boyer JM, Shafritz D, Fausto N, Harvey A, eds. *The Liver: Biology and Pathobiology, 5*. Chichester: Wiley; 2009:349–358.
32. Wakabayashi Y, Lippincott-Schwartz J, Arias IM. Intracellular trafficking of bile salt export pump (ABCB11) in polarized hepatic cells: constitutive cycling between the canalicular membrane and rab11-positive endosomes. *Mol Biol Cell*. 2004;15: 3485–3496.
33. Wang W, Soroka CJ, Mennone A, et al. Radixin is required to maintain apical canalicular membrane structure and function in rat hepatocytes. *Gastroenterology*. 2006;131:878–884.
34. Huang L, Smit JW, Meijer DK, et al. Mrp2 is essential for estradiol-17beta(beta-D-glucuronide)-induced cholestasis in rats. *Hepatology*. 2000;32:66–72.