RESEARCH COMMUNICATION

Stimulation of ATP secretion in the liver by therapeutic bile acids

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ATP receptors are ubiquitously expressed and are potential targets for the therapy of a number of disorders. However, delivery of ATP or other nucleotides to specific tissues is problematic, and no pharmacological means to stimulate the release of endogenous ATP has been described. We examined the effects of the bile acid ursodeoxycholic acid (UDCA) on ATP release into bile, since this bile acid is the only agent known to be of therapeutic benefit in secretory disorders of the liver, and since its mechanism of action is not established. Both UDCA and its taurine conjugate stimulated secretion of ATP by isolated rat hepatocytes, and produced measurable increases in ATP in bile of isolated rat liver. Perfusion of ATP into microdissected bile-duct segments induced Ca^{2+} signalling in bile-duct epithelia, while perfusion of bile acid did not. Thus UDCA may promote bile flow by inducing hepatocytes to release ATP into bile, which then stimulates fluid and electrolyte secretion by bile-duct epithelia downstream via changes in cytosolic Ca^{2+}. Moreover, these findings demonstrate the feasibility of using pharmacological means to induce secretion of endogenous ATP. Since the liver and other epithelial organs express luminal ATP receptors, these findings more generally suggest that a mechanism exists for pharmacological activation of this paracrine signalling pathway. This strategy may be useful for treatment of cystic fibrosis and other secretory disorders of the liver and other epithelial tissues.

Key words: bile duct, cholestasis, cystic fibrosis, P2Y receptor, ursodeoxycholic acid.

INTRODUCTION

Members of the P2 class of ATP receptors are expressed in virtually every type of tissue [1,2]. Specific subtypes of P2X and P2Y receptors regulate a number of physiological functions, ranging from secretion to platelet aggregation to fertility [3–7]. Thus P2 receptors may be potential targets for the therapy of a variety of disorders. Since epithelial organs such as lung [4,8], liver [9], and salivary glands [7] express apical P2Y receptors that link to fluid and electrolyte secretion, stimulation of these receptors may provide a novel means for treatment of secretory disorders. Indeed, UTP in aerosol form has been used to try to stimulate P2Y receptors in the respiratory tract of patients with cystic fibrosis [4]. However, it has been difficult to deliver sufficient amounts of ATP or other nucleotides to specific tissues. Although a number of cell types are capable of secreting ATP [10–12], no pharmacological means to stimulate the release of endogenous ATP has been described. The bile acid ursodeoxycholic acid (UDCA) is known to be beneficial for the hepatic manifestations of cystic fibrosis [13], as well as for other secretory disorders of the liver [14,15], yet its mechanism of action remains unclear [16]. Therefore we examined the effects of UDCA on ATP release into bile.

METHODS

Animals and materials

Male Sprague–Dawley rats (180–250 g) were used for all experiments except microperfusion of intrahepatic bile-duct units (IBDUs), in which male Fischer 344 rats (225–250 g) were used. ATP and all bile acids were obtained from Sigma (St Louis, MO, U.S.A.), and fluo-4 acetoxymethyl ester (fluo-4/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were of the highest quality commercially available.

Isolation of hepatocytes

Isolated rat hepatocytes were prepared in the Cell Isolation Core Facility of the Yale Liver Center as described previously [12]. Rat livers were perfused with collagenase-containing medium, then excised, minced, and passed through serial nylon-mesh filters, and the resultant cells were washed. These cells were suspended in Leibovitz L-15 medium containing 10 % (v/v) fetal-calf serum, 50 units of penicillin and 50 mg of streptomycin/ml, and plated on to glass coverslips. Cells were incubated at 37 °C and used 2–6 h after plating. The viability of hepatocytes, measured by Trypan Blue exclusion, exceeded 90 % at 2 h after plating.

Isolated-perfused-liver studies

Liver perfusions were performed in the Perfusion Core Facility of the Yale Liver Center as described previously [17]. Rats were anaesthetized with pentobarbital (50 mg/kg body wt) and the bile duct was cannulated. The portal vein was then cannulated with an intravenous catheter and the liver was perfused at 25 ml/min with oxygenated Krebs–Ringer bicarbonate (KRB) buffer containing heparin. After cannulation of the inferior vena cava, the liver was transferred into a temperature-controlled

Abbreviations used: UDCA, ursodeoxycholic acid; IBDU, intrahepatic bile-duct unit; fluo-4/AM, fluo-4 acetoxymethyl ester; KRB, Krebs–Ringer bicarbonate; TUDCA, tauroursodeoxycholic acid; TCA, taurocholic acid (note that, in this paper, TCA does not have its more usual meaning of trichloroacetic acid or tricarboxylic acid); TDHCA, taurodehydrocholic acid; TLCA, taurolithocholic acid; Ca_i^{2+}, cytosolic Ca^{2+}; ABC, ATP binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator.

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perfusion chamber and perfused in a non-recirculating (i.e. single-pass) fashion at 40 ml/min with KRB buffer. The buffer was gassed continuously with a mixture of O_2/CO_2 (19:1) and maintained at 37 °C by monitoring the temperature with a thermister probe inserted between the lobes of the liver. Bile flow was measured gravimetrically in pre-tared tubes and perfusion pressure was monitored continuously. Biliary ATP was measured by luminometry as described below, and bile samples obtained for this purpose were collected and maintained on ice until they were analysed. The viability of each liver preparation was ascertained by monitoring perfusion pressure and O_2 consumption during the course of the experiment, and by determining Trypan Blue distribution upon completion.

Measurement of ATP

ATP was measured by the luciferan/luciferase reaction using a kit (Sigma). This reaction is highly specific for detection of ATP rather than other nucleotides [18,19]. In isolated hepatocyte studies, 5×10^6 cells/ml were mixed with 25 μM bile acid in a Hepes-based buffer for 5 min at 37 °C, then the cells were placed on ice, the supernatant was removed, and the ATP concentration in the supernatant was determined by luminometry. Standard curves relating ATP concentration to luminescence were log–linear over the range 1 nM–1 μM ($r = 0.9957$; $P < 0.00001$). These ATP measurements were not altered by addition of the bile-acid-binding agent cholestyramine (50 mg/ml) to the supernatant, indicating that the measurements were not affected by the presence of bile acids. ATP in bile was measured as described previously [20]. Briefly, bile was collected in 5 min increments directly into an equal volume of 0.8 M perchloric acid on ice, then protein was removed by addition of an equal volume of 1 M K_2HPO_4. The ATP concentration in the resulting solution was measured immediately afterwards by luminometry.

Microperfused-bile-duct studies

Cytosolic Ca^{2+} was measured in bile-duct epithelia within isolated IBDUs using microspectrofluorimetry [21]. IBDUs were isolated from normal rat liver, then individual isolated IBDUs were placed in a specially designed, temperature-controlled chamber mounted on the stage of an inverted fluorescence microscope (Nikon Eclipse TE300). Concentric glass pipettes were attached to a custom-built microperfusion apparatus and used to position and perfuse the IBDUs [21]. IBDUs were perfused at rates of 10–100 nl/min while the external surface of the perfused IBDUs were simultaneously bathed in a buffer maintained at 37 °C. The perfused IBDUs ranged in luminal diameter from 100 to 125 μm and in length from 1.0 to 1.5 mm. Viability was assessed by Trypan Blue exclusion. IBDUs were microperfused with the Ca^{2+}-sensitive fluorescent dye fluo-4/AM (2 μM) for 30 min to load the dye into bile-duct epithelia. The fluo-4 was excited at 480 nm using a 100 W mercury lamp. Fluorescence emission through a 50–100-μm-diameter circular spot at the distal end of IBDUs was detected through a 505-nm dichroic mirror and a 535/40-nm barrier filter using an H5784 photosensor module (Hamamatsu Photonics, Bridgewater, NJ, U.S.A.). The signal was digitized and collected at a rate of 5–10 samples/s, then low-pass-filtered for noise reduction. The perfused IBDUs were allowed to equilibrate for 30–40 min before starting each experiment, then ductules were perfused with either tauroursodeoxycholic acid (TUDCA) or ATP. A total of 12 rats were used for IBDU studies; three or four rats were used in each set of experiments, to obtain five to seven IBDUs for each experimental condition. Fluorescence measurements (F) were scaled by initial fluorescence (F_0), then expressed as $100\,\% \times (F/F_0)$.

RESULTS AND DISCUSSION

UDCA is rapidly conjugated to TUDCA in the liver [22], so the effects of both of these bile acids on isolated rat hepatocytes were examined. UDCA and TUDCA each induced isolated rat hepatocytes to release ATP, while three other bile acids had minimal or no effects (Figure 1). One of these bile acids, taurocholic acid (TCA), is the predominant bile acid in the rat, and stimulates bile flow to a similar extent as TUDCA [23]. The second bile acid, taurodehydrocholic acid (TDHCA), stimulates bile flow even more potently than TCA [24]. The third bile acid, taurolithocholic acid (TLCA), inhibits rather than stimulates bile flow and can be hepatotoxic [25], but TLCA and UDCA both increase cytosolic Ca^{2+} (Ca_i^{2+}) in hepatocytes much more effectively than most other bile acids [26]. Therefore the effects of UDCA and its taurine conjugate on ATP release appear to be specific for these particular bile acids, and are not merely related to their effects on bile flow or Ca_i^{2+} signalling. Dose–response curves (Figure 2, upper, middle and lower panels) revealed that both of these bile acids stimulate ATP release in a bimodal fashion, exerting their maximum effect at low micromolar concentrations. In addition, cell viability was not impaired by incubation with either UDCA or TUDCA, even at a concentration of 1 mM (results not shown). These findings suggest that ATP secretion is not merely a non-specific effect resulting from the detergent-like action that some bile acids can exert at higher concentrations. For comparison, typical serum levels of UDCA and TUDCA are $\approx 10\,\mu$M in non-cirrhotic patients being treated with UDCA, 30–40 μM in cirrhotic patients and $\approx 0.1\,\mu$M in untreated individuals [22]. Thus maximal ATP release from hepatocytes is induced by concentrations of UDCA and TUDCA that occur *in vivo*.

Hepatocytes are in contact with both blood (across their sinusoidal membrane) and bile (across their canalicular membrane), so we investigated whether UDCA and TUDCA induce hepatocytes to secrete detectable amounts of ATP into bile in particular. The effects of these and other bile acids on biliary ATP were measured in the isolated perfused rat liver (Table 1 and Figure 3). Since nucleotides are normally found in bile [20],

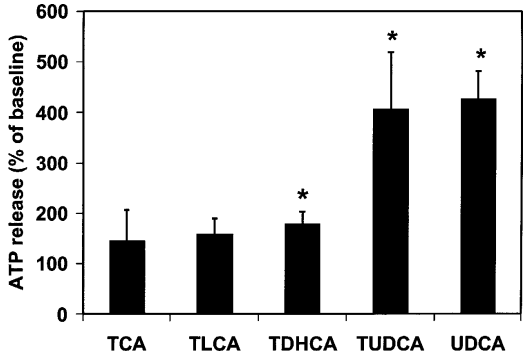

Figure 1 Effect of bile acids on ATP release from isolated rat hepatocytes

Hepatocytes were incubated with each bile acid (25 μM) for 5 min at 37 °C, then ATP in the supernatant was measured by luminometry using the luciferase reaction. Results were normalized by ATP release measured simultaneously from untreated hepatocytes, and represent means $\pm$ S.E.M. for five separate experiments (*$P < 0.05$). UDCA and its taurine conjugate TUDCA stimulate ATP release from isolated rat hepatocytes. No such effect is seen with TCA or TLCA, and TDHCA increases ATP release only minimally.

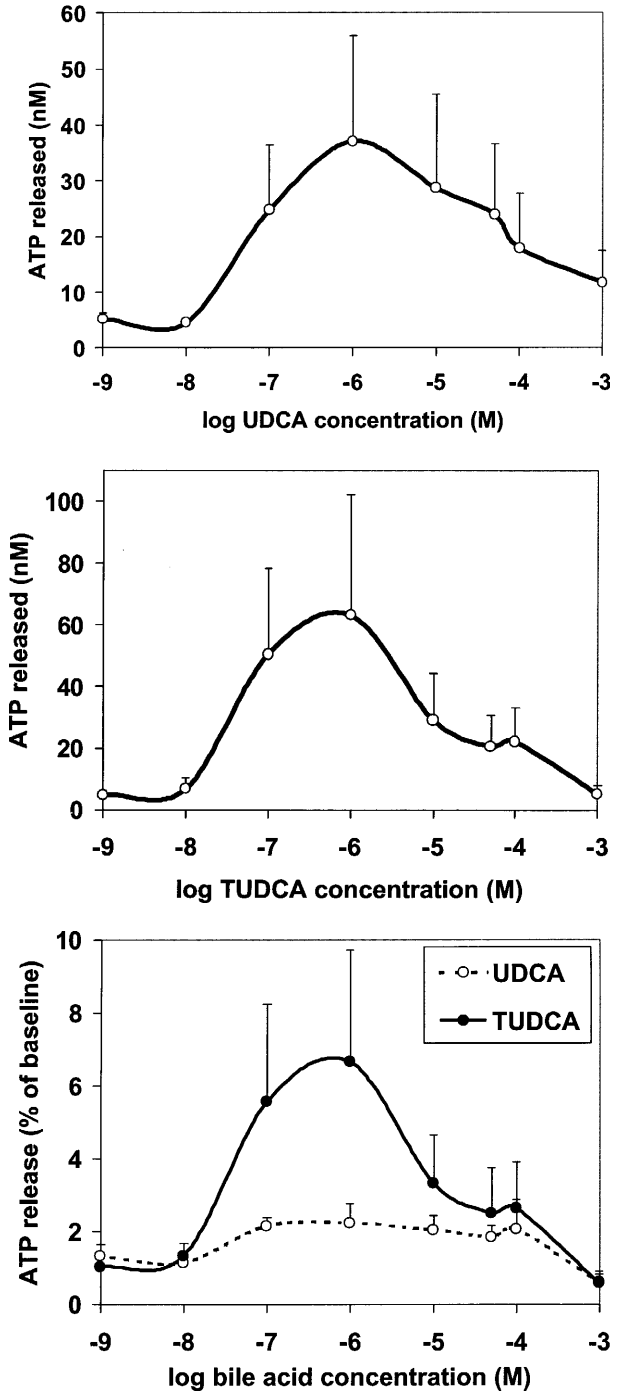

Figure 2 Concentration-dependent effects of UDCA and TUDCA on ATP release

Upper panel: UDCA-induced release of ATP from isolated hepatocytes is maximal at 1–10 μM. Isolated hepatocytes were incubated with each concentration of UDCA for 5 min, then ATP in the supernatant was measured by luminometry. Middle panel: TUDCA-induced release of ATP from isolated hepatocytes is maximal at 1 μM. Isolated hepatocytes were incubated with each concentration for 5 min, then ATP in the supernatant was measured by luminometry. Lower panel: normalized ATP release. Results from UDCA and TUDCA experiments were scaled by baseline ATP release in each experiment to minimize variability during comparisons. Results represent means $\pm$ S.E.M. for four separate experiments in which each data point was measured in triplicate.

biliary ATP was determined both before and after bile acid administration to control for basal release of ATP (Table 1). TUDCA increased biliary ATP by $160 \pm 64\,\%$ (mean $\pm$ S.E.M.) relative to baseline ($P < 0.05$), whereas UDCA increased biliary ATP by $278 \pm 69\,\%$ ($P < 0.05$). TCA significantly increased biliary ATP as well, but by only $80 \pm 22\,\%$ ($P < 0.05$). Since hepatocytes express ecto-ATPases [27], those bile acids that increase bile flow might increase biliary ATP simply because of decreased hydrolysis of ATP, rather than increased ATP secretion. To investigate this, we examined the relationship between bile-acid-induced bile flow and biliary ATP (Figure 3). Indeed, in livers perfused with TCA, TDHCA or TLCA, there was a linear relationship between bile flow rate and biliary ATP ($r = 0.82$, $P = 0.001$; Figure 3). However, both UDCA and TUDCA increased biliary ATP beyond the amount that could be accounted for by increased bile flow (Figure 3). Together, these findings suggest that these two bile acids induce hepatocytes to secrete ATP into bile.

Table 1 Effects of various bile acids on ATP in bile

Each bile acid was infused into isolated perfused rat livers at a concentration of 50 μM, except TLCA, which was infused at a concentration of 25 μM to avoid hepatotoxicity. Bile flow and biliary ATP were measured both before and after infusion of each bile acid, and biliary ATP is expressed as a percentage of baseline to adjust for individual variations in biliary ATP before infusion of bile acid. Biliary ATP is significantly (*$P < 0.05$) increased by infusion of either TUDCA ($n = 5$) or UDCA ($n = 4$). TCA ($n = 5$) increases biliary ATP as well, but to a lesser extent. Neither TLCA ($n = 5$) nor TDHCA ($n = 2$) increases biliary ATP relative to baseline. Note the progressive increase in ATP from TLCA to TCA to TDHCA, which corresponds to the relative ability of each of these bile acids to induce bile flow. Values are means $\pm$ S.E.M.

Bile acid	Biliary ATP
TUDCA	260 ± 64*
UDCA	378 ± 69*
TLCA	80 ± 20
TCA	180 ± 22*
TDHCA	239 ± 100

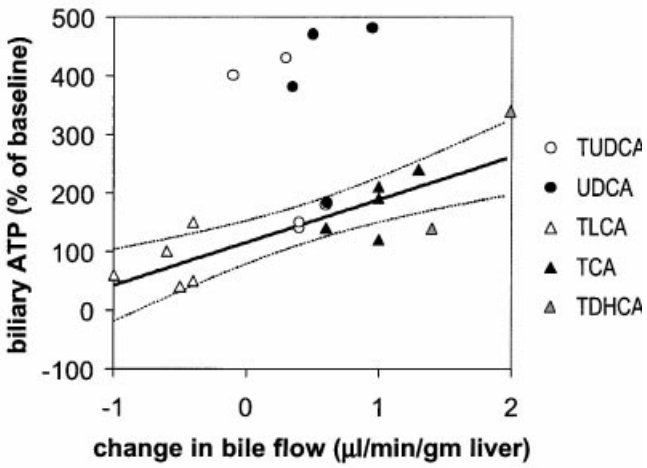

Figure 3 UDCA and its taurine conjugate increase ATP in bile

Isolated rat livers were perfused either with UDCA, TUDCA, TCA, TDHCA or TLCA. Each bile acid was infused at a concentration of 50 μM, except TLCA, which was infused at a concentration of 25 μM to avoid hepatotoxicity. Bile flow and biliary ATP were measured both before and after infusion of each bile acid, and biliary ATP is expressed as a percentage of baseline to adjust for individual variations in biliary ATP before infusion of bile acid. This graph illustrates the relationship between biliary ATP and bile flow, to adjust for bile acid-induced changes in bile flow. Biliary ATP and bile flow are linearly related for TCA, TDHCA and TLCA (the continuous line is the linear regression curve; the broken lines are 95% confidence intervals). However, UDCA- and TUDCA-induced ATP release is far greater than expected in five of the eight livers, since these data points lay well above the 95% confidence region.

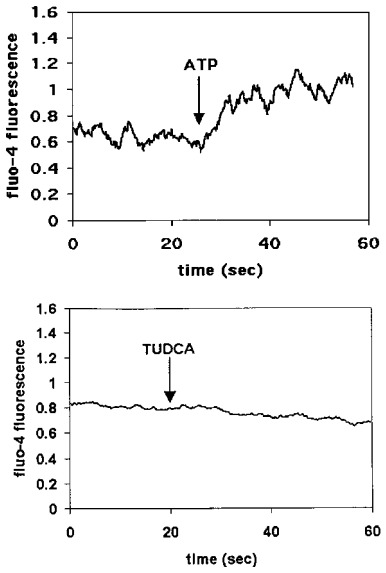

Figure 4 Biliary ATP induces Ca_i^{2+} signalling in bile-duct epithelia, while TUDCA in bile does not

Upper panel: luminal perfusion with ATP (500 nM) increases Ca_i^{2+} in bile-duct epithelia ($P < 0.05$ relative to epithelia in ducts perfused with buffer alone). The trace is representative of that observed in five IBDUs. Lower panel: luminal perfusion with TUDCA (100 μM) does not increase Ca_i^{2+} in bile-duct epithelia ($P = 0.90$ relative to epithelia in ducts perfused with buffer alone). The trace is representative of that observed in seven IBDUs perfused with 100–200 μM TUDCA.

UDCA is known to stimulate both bile flow and bicarbonate excretion [28,29], although the mechanism for this is not established. The bile-duct epithelia play a role in biliary bicarbonate excretion [9,30–32], so we examined whether TUDCA acts directly or indirectly on these cells. To investigate this, intrahepatic segments of the biliary tree were microdissected, then microperfused with either TUDCA (100–200 μM) or ATP (500 nM), concentrations that occur *in vivo* [20,22]. This particular ATP concentration was chosen for several other reasons as well. Although the maximal ATP concentration released from populations of isolated hepatocytes was 40–60 nM (Figure 2), the number of hepatocytes and the volume of buffer in which they were incubated were arbitrary, so that ATP concentration does not necessarily reflect the ATP concentration in bile. In fact, the average concentration of nucleotides is 400 nM in rat bile, and is as great as 5 μM in man [20]. Furthermore, biliary ATP increases Ca_i^{2+} in a concentration-dependent fashion in bile-duct epithelia, as ATP in bile increases from 100 nM to 100 μM [9]. Therefore 500 nM ATP was used for microperfusions, because it represents a conservative estimate of what would be expected *in vivo*. Ca_i^{2+} was monitored during microperfusions, since both TUDCA and ATP can increase Ca_i^{2+} in some epithelia [12,26], and since Ca_i^{2+} signalling links to activation of anion channels and bicarbonate secretion in bile-duct epithelia in particular [9,30,33]. Fluorescence of the Ca^{2+} dye fluo-4 within bile-duct epithelia was used to monitor Ca_i^{2+}. ATP induced a rapid, transient $23 \pm 3\%$ increase in fluorescence ($P < 0.05$), whereas TUDCA did not (Figure 4). These findings are consistent with the observation that ATP stimulates apical P2Y receptors and activates apical chloride channels in a polarized bile-duct cell line [34] and bicarbonate secretion in bile ducts [9]. Moreover, these findings suggest that TUDCA affects bile composition and flow through the paracrine actions of ATP released by hepatocytes, rather than through a direct effect of TUDCA on bile-duct epithelia.

ATP activates apical anion channels in the epithelia lining the ducts of a number of organs, including liver as well as lung, pancreas, submandibular gland and testes [7,34–37]. These actions are thought to result from stimulation of apical P2Y receptors, which increases Ca_i^{2+} and thus activates Ca^{2+}-dependent chloride channels [38]. Apical P2Y receptors are more effective than basolateral P2Y receptors in increasing Ca_i^{2+} and stimulating ductular bicarbonate secretion in the liver in particular [9]. Molecular and pharmacological evidence suggests that bile-duct epithelia express P2Y1, P2Y2, P2Y4 and P2Y6 ATP receptors on their apical membrane, so bile-duct cells could respond not only to ATP, but to ADP, UTP or UDP in bile as well [9]. In the present study we only measured ATP release, although some cells are known to release UTP as well [39]. Measurement of this and other nucleotides is much less straightforward than measurement of ATP, however [39]. Thus the present study does not address the question of whether uridine nucleotides are present in bile, or if their presence is affected by bile acids. Although the present study demonstrates that TUDCA and UDCA induce hepatocytes to secrete ATP into bile, the mechanism by which this occurs remains unclear. There is evidence that ATP binding cassette (ABC) proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR) and Mdr1 can regulate ATP release from cells [40–42], although this is controversial [43]. However, the hepatocyte bile-salt export pump BSEP is an ABC protein as well [44], and is likely to transport both UDCA and TUDCA into bile [44]. Indirect evidence furthermore suggests that BSEP may in part regulate release of ATP from hepatocytes [45,46]. However, further work is needed to determine directly whether BSEP is involved in ATP release, as well as whether TUDCA or UDCA would preferentially affect this function of BSEP.

The present results suggest that UDCA and TUDCA induce hepatocytes to release ATP into bile. Although ATP may exert a number of potential effects on bile-duct epithelia, here we only examined effects on Ca_i^{2+} signalling. Ca_i^{2+} specifically induces secretion of bicarbonate in bile-duct epithelia, which likely results from serial activation of Ca^{2+}-dependent chloride channels, then chloride $\leftrightarrow$ bicarbonate exchange [30]. UDCA is known to induce secretion of bicarbonate into bile [28], so the present results suggest that this may result in part from UDCA- and TUDCA-induced secretion of ATP by hepatocytes, followed by ATP-induced bicarbonate secretion by bile-duct epithelia. Since Ca^{2+}-dependent chloride channels in bile-duct cells and other epithelia are distinct from the CFTR, it has been hypothesized that apical nucleotides could be used to bypass CFTR defects in patients with cystic fibrosis [4,8]. UTP in aerosol form thus has been used to treat pulmonary manifestations of cystic fibrosis, but with limited efficacy [47], perhaps due to degradation of the nucleotide before it can reach its target tissue. Stimulation of endogenous release of ATP would provide a novel alternative to activate non-CFTR chloride channels, but no pharmacological means to stimulate ATP secretion has been described until now. Hepatocytes are able to secrete ATP in high enough concentrations to stimulate nearby bile-duct epithelia [12], and the current work suggests that UDCA may be one agent that induces physiologically relevant ATP secretion. Moreover, it is already established that UDCA improves liver function in cholestatic liver disorders such as primary biliary cirrhosis [14,15] and cystic fibrosis [13]. Further work will be needed to demonstrate whether the therapeutic effect of this unique bile acid is mediated in part by stimulation of ATP release, and to determine the mechanism by which ATP release occurs.

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