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Ursodeoxycholic acid attenuates colonic epithelial secretory function

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KEY POINTS SUMMARY

• Although diarrhoeal diseases represent a great health and economic burden to society, therapeutic options remain limited.

• While several bile acids are known to stimulate epithelial Cl− secretion, the major driving force for fluid secretion in the intestine, the effects of ursodeoxycholic acid (UDCA) on epithelial transport function are not known.

• We report that in contrast to other bile acids, UDCA exerts antisecretory actions on colonic epithelial cells in vitro.

• In contrast, in vivo administration of UDCA enhances epithelial secretory function, an affect we ascribe to being due to its bacterial metabolism to lithocholic acid (LCA). In keeping with this hypothesis, in vivo administration of a metabolically stable analogue of UDCA, 6α-methyl-UDCA, was antisecretory.

• Our findings reveal a novel antisecretory effect of UDCA and suggest that metabolically stable analogues the bile acid may be useful for development as a new class of anti-diarrhoeal drug.

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Dihydroxy bile acids, such as chenodeoxycholic acid (CDCA), are well-known to promote colonic fluid and electrolyte secretion, thereby causing diarrhoea associated with bile acid malabsorption. However, CDCA is rapidly metabolised by colonic bacteria to ursodeoxycholic acid (UDCA), the effects of which on epithelial transport are poorly characterised. Here, we investigated the role of UDCA in regulation of colonic epithelial secretion. Cl⁻ secretion was measured across voltage-clamped monolayers of T₈₄ cells and muscle-stripped sections of mouse or human colon. Cell surface biotinylation was used to assess abundance/surface expression of transport proteins. Acute (15 min) treatment of T₈₄ cells with bilateral UDCA attenuated Cl⁻ secretory responses to the Ca²⁺ and cAMP-dependent secretagogues, carbachol (CCh) and forskolin (FSK) to 14.0 ± 3.8% and 40.2 ± 7.4% of controls, respectively (n = 18; p < 0.001). An investigation of molecular targets involved revealed that UDCA acts by inhibiting Na⁺/K⁺-ATPase activity and basolateral K⁺ channel currents, without altering their cell surface expression. In contrast, intraperitoneal administration of UDCA (25 mg/kg) to mice enhanced agonist-induced colonic secretory responses, an effect we hypothesised to be due to bacterial metabolism of UDCA to lithocholic acid (LCA). Accordingly, LCA (50–200 μM) enhanced agonist-induced secretory responses in vitro and a metabolically stable UDCA analogue, 6α-methyl-UDCA (6α-MUDCA), exerted antisecretory actions in vitro and in vivo. In conclusion, UDCA exerts direct antisecretory actions on colonic epithelial cells and metabolically stable derivatives of the bile acid may offer a new approach for treating intestinal diseases associated with diarrhoea.

**Abbreviations:** 4-PB, 4-phenylbutyrate; 6α-MUDCA, 6α-methyl-UDCA; CCh, carbachol; CDCA, chenodeoxycholic acid; CFTR, cystic fibrosis transmembrane conductance regulator; FSK, forskolin; Isc, short-circuit current; LCA, lithocholic acid; UDCA, ursodeoxycholic acid
INTRODUCTION

Appropriate regulation of intestinal epithelial fluid and electrolyte transport is important for normal digestive function, mucosal protection, and whole body fluid homeostasis. Under normal circumstances fluid absorption, driven mainly by Na\(^+\) absorption, predominates. However, fluid secretion, driven by Cl\(^-\) secretion, also occurs and is important for lubricating and protecting the mucosa (Barrett & Keely, 2000). The balance between absorption and secretion is highly dynamic and is regulated by an array of neuronal, immunological, and hormonal messengers. Many intestinal disorders exist that perturb the balance between absorption and secretion leading to the onset of diarrhoea. While infectious diarrhoea is responsible for ~ 2 million child deaths each year in developing countries, common causes of diarrhoea in Western societies include intestinal pathogens, inflammatory diseases, irritable bowel syndrome, and digestive and metabolic disorders. However, even though the social and economic burdens of such diseases are immense (Sandler et al., 2002), there are still relatively few safe and effective therapeutic options for their treatment.

At the molecular level, intestinal fluid secretion is co-ordinated by the activity of several transport proteins (Barrett & Keely, 2000). Energy for the process comes from the activity of basolateral Na\(^+\)/K\(^+\) ATPase pumps which maintain low intracellular Na\(^+\) concentrations by pumping Na\(^+\) from the cell in exchange for K\(^+\). This provides a favourable gradient for basolateral Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter activity, which carries Na\(^+\) into the cell along with K\(^+\) and 2Cl\(^-\). K\(^+\) does not accumulate intracellularly, since it exits through the basolateral K\(^+\) channels, KCNN4 and KCNQ1. Thus, the concerted activity of these basolateral transporters specifically increases intracellular Cl\(^-\) concentrations so that a gradient exists for its exit when channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR), in the apical membrane open. Since Cl\(^-\) secretion drives intestinal fluid secretion, these transport proteins represent excellent targets for the development of drugs to treat secretory diarrhoea.
Although classically known for their roles in lipid digestion, bile acids are becoming increasingly recognised as important regulators of intestinal epithelial function. The primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesised in the liver, stored in their conjugated forms in the gallbladder, and are released into the duodenum upon ingestion of food. As they pass through the small intestine, bile acids facilitate the digestion and absorption of lipids, and are reabsorbed in the terminal ileum to be recycled back to the liver. Normally, this enterohepatic circulation of bile acids is extremely efficient with < 5% of circulating bile acids entering into the colon with each cycle. Here, they are metabolised by resident bacteria, primarily by deconjugation, dehydroxylation and epimerisation, to yield secondary bile acids. Cholic acid is metabolised to deoxycholic acid (DCA), while CDCA is metabolised to ursodeoxycholic acid (UDCA), which is then further metabolised to lithocholic acid (LCA) (Hofmann, 2009).

Previous studies have shown that conjugated and unconjugated bile acids rapidly induce colonic epithelial Cl\(^-\) secretion (Volpe & Binder, 1975; Chadwick et al., 1979; Dharmsathaphorn et al., 1989; Potter et al., 1991; Gelbmann et al., 1995; Mauricio et al., 2000; Venkatasubramanian et al., 2001; Moschetta et al., 2003). While there are considerable species differences in the potencies of different bile acids, it is clear that in humans, prosecretory effects occur only when bile acid levels enter the pathophysiological range (Mekhjian & Phillips, 1970). Such prosecretory effects are thought to underlie the diarrhoea that is associated with conditions of bile acid malabsorption. Mechanisms by which bile acids stimulate Cl\(^-\) secretion have been well-studied in vitro, with both Ca\(^{2+}\)- and cAMP-dependent mechanisms being implicated (Dharmsathaphorn et al., 1989; Devor et al., 1993; Huang et al., 1995; Moschetta et al., 2003). Furthermore, it appears that bile acids can exert their prosecretory effects either by acting directly on epithelial cells themselves, or through stimulation of regulatory cells within the mucosa (Karlstrom et al., 1986; Gelbmann et al., 1995).

Notably, there are strict structure-activity requirements for bile acids in promoting secretion, with only dihydroxy bile acids, such as DCA and CDCA, being effective
(Chadwick et al., 1979; Gordon et al., 1979). However, UDCA, a dihydroxy bile acid that is formed by bacterially-mediated epimerisation of the 7α-OH group of CDCA, and which is also in widespread use as a therapeutic in liver disease, has been reported to be devoid of prosecretory actions in vitro (Gelbmann et al., 1995; Keely et al., 2007). In the current study, we set out to more fully investigate effects of UDCA on colonic epithelial secretory function.
METHODS

**Chemicals:** CDCA-Na$^+$ salt and LCA-Na$^+$ salt were obtained from Calbiochem (San Diego, CA, USA). UDCA-Na$^+$ salt, carbachol, forskolin, amiloride, phloridzin, tetrodotoxin, nystatin, and amphotericin B were from Sigma-Aldrich Inc. (Gillingham, UK). Stock solutions of bile acids were prepared in deionised H$_2$O. Sulfo-NHS-Biotin was from Pierce Biotechnology, Rockford, IL, USA. Anti-Na$^+/K^+$ ATPase $\alpha_1$ subunit antibodies (mouse) and anti-KCNN4 antibodies (rabbit) were obtained from AbCam (Cambridge, UK) and anti- $\beta$-actin was from Sigma-Aldrich (St.Louis, MO). All other reagents were of analytical grade.

**Cell Culture:** $T_{84}$ colonic epithelial cells were cultured in DMEM/Hams F12 media (1:1) supplemented with 5% newborn calf serum (Keely et al., 2007). For electrophysiological studies, 5 x 10$^5$ cells were seeded onto 12 mm Millicel HA Transwells (Millipore, Bedford, MA, USA). For western blot and surface biotinylation experiments, 1 x 10$^6$ cells were seeded onto 30 mm Millicell HA Transwells. Cells seeded onto filters were cultured for 10–15 days before use. Under these conditions, $T_{84}$ cells develop the polarised phenotype of native epithelial cells and vectorially secrete Cl$^-$ in a basolateral to apical direction.

**Human tissues:** Resected colonic tissue was obtained from adult patients undergoing colorectal surgery in Beaumont Hospital. Patients agreed to participate by written informed consent and this study was approved by Beaumont Hospital Medical Ethics Committee. Normal colonic mucosa was identified by pathologists and specimens were taken at least 3 cm clear of the tumour and resection margins. Patients receiving chemo/radiotherapy, or those with a history of inflammatory bowel disease, were excluded. Fourteen patients (10 male), with a median age of 63 (42 - 85), were included, none of whom were taking bile acid sequestrants or UDCA at the time of surgery.

**Animals:** Male C57 BL6 mice aged 6–12 weeks were used. Animals were maintained in an environmentally-controlled facility (12h light/12h dark cycle) with access to food and
water *ad libitum*. Animals were fed 2018 Teklad Global 18% Protein Rodent Diet (Harlan Laboratories Inc). Experiments were approved by the RCSI Ethics Committee. UDCA (25 mg/kg), 6α-MUDCA (25 mg/kg), or endotoxin-free phosphate buffered saline as vehicle, were injected intraperitoneally, and mice were sacrificed 4 hrs later by cervical dislocation.

*Electrophysiological Measurements:* T₈₄ monolayers were washed in serum-free medium and allowed to equilibrate for 1 hr. Cells were mounted in Ussing chambers (aperture = 0.6cm²), voltage-clamped to zero potential difference, and monitored for changes in short-circuit current (Iₛₖ) using a VCC-MC8 voltage clamp (Physiological Instruments, San Diego, CA). Under such conditions, agonist-induced changes in Iₛₖ are wholly reflective of changes in Cl⁻ secretion (Cartwright *et al.*, 1985). Iₛₖ measurements were carried out in Ringer’s solution containing (in mM): 140 Na⁺, 5.2 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 119.8 Cl⁻, 25 HCO₃⁻, 0.4 H₂PO₄⁻, 2.4 HPO₄²⁻ and 10 glucose, at pH 7.4 and aerated with 95% O₂/5% CO₂. Results were expressed as ΔIₛₖ (µA/cm²) or as a percentage of control responses.

Apical Cl⁻ currents were measured as previously described (Rochwerger *et al.*, 1994). Briefly, cell monolayers were mounted in Ussing chambers under an apical to basolateral Cl⁻ gradient (119.8–4.8 mM), established by replacing NaCl in the basolateral solution with equimolar Na-gluconate. Monolayers were basolaterally permeabilised with nystatin (100 µg/ml), and after a 35 min re-equilibration period, cells were stimulated with forskolin (FSK; 10 µM). Under these conditions, changes in Iₛₖ reflect apical Cl⁻ currents (Iₘ₃).

Basolateral K⁺ currents were analyzed as previously described (Kirk & Dawson, 1983). Voltage-clamped T₈₄ monolayers were apically permeabilised using amphotericin B (50 µM). A K⁺ gradient (123.2–5.2 mM) was then created across the basolateral membrane by addition of a high-K⁺ (123.2 mM) Ringer’s solution, in which NaCl is substituted with K⁺-gluconate, to the apical reservoir. Ouabain (100 µM) was added basolaterally to
inhibit Na\(^+\)/K\(^+\)-ATPase activity. Under these conditions, changes in \(I_{sc}\) are reflective of basolateral K\(^+\) currents (\(I_K\)).

Na\(^+\)/K\(^+\)-ATPase activity was measured as described by Lam et al. (Lam et al., 2003). T\(_{84}\) monolayers were bathed bilaterally in a low-Na\(^+\) (25 mM) Ringer’s solution, where NaCl was substituted with equimolar N-methyl-d-glucamine (NMDG)-Cl\(^-\). Apical membranes were permeabilised with amphotericin B (50 \(\mu\)M), and under these conditions changes in \(I_{sc}\) reflect Na\(^+\)/K\(^+\)-ATPase activity.

Sections of isolated human or mouse colonic mucosa were obtained by blunt micro-dissection of underlying muscle layers. Tissues were mounted in Ussing chambers (window area = 0.3 cm\(^2\) for mouse and 0.5 cm\(^2\) for human specimens) and bathed in Ringers solution for measurements of \(I_{sc}\). Tissues were allowed to equilibrate for 30 min before addition of pharmacological reagents.

For measurements of sodium glucose co-transporter (SGLT-1) activity in mice, whole thickness sections of proximal jejunum were mounted in Ussing chambers and bathed apically in glucose-free Ringers, containing mannitol (5 mM), and basolaterally in normal Ringers solution. To stimulate SGLT-1 activity, 25 mM glucose was added apically, with 25 mM mannitol being added basolaterally at the same time to maintain isosmolarity, and changes in \(I_{sc}\) were measured. 1mM phloridzin was used to confirm increases in \(I_{sc}\) were due to SGLT-1 activity (Grubb, 1995).

Measurements of Caecal Bile Acids: Caecal contents were collected from treated and control animals and stored in isopropanol at -20\(^\circ\)C. Caecal bile acid levels were measured using HPLC-ES-MS/MS and the qualitative caecal bile acid composition was expressed as % total bile acid content, as previously described (Tadano et al., 2006).

Cell Surface Biotinylation: The protocol used was based on one previously published (Del Castillo et al., 2005). Following treatment, cells were washed with ice-cold PBS. Freshly prepared biotinylation buffer (1mg/ml Sulfo-NHS-Biotin in PBS) was added
basolaterally and cells were incubated at 4°C for 15 minutes on a rotating platform after which the buffer was replenished. After another 15 mins, cells were washed, incubated with quenching reagent (100 mM glycine), lysed (Triton lysis buffer; 45 mins), and centrifuged (15,300 g, 6 mins). After normalising for protein concentration, supernatants were then precipitated overnight on a rotator at 4°C with 100 µL of streptavidin–agarose beads (Pierce Biotechnology, Rockford, IL, USA). Biotinylated proteins were detected by western blotting.

**Western Blotting:** T84 monolayers were washed with ice-cold PBS and lysed (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris Base, 1 Complete mini EDTA-free protease inhibitor tablet, 0.1 mg/ml PMSF and 1 mM Na₃VO₄). Lysates were sonicated, centrifuged (15,300 g; 10 min; 4°C), and the pellet discarded. After normalising for protein content, 2x gel loading buffer (50 mM Tris HCl, 100 mM DTT, 40% glycerol, 4% SDS) was added, and samples were then separated by SDS-PAGE, followed by transfer to PVDF membranes. Membranes were preblocked in 5% blocking buffer (Marvel in Tris-buffered saline with 1% Tween (TBST)) for 60 min at room temperature, followed by incubation with primary antibody in blocking buffer overnight at 4°C. The primary antibodies employed were anti-Na⁺/K⁺ ATPase α₁ subunit antibodies (mouse, 1:10,000 dilution), anti-KCNN4 antibodies (rabbit; 1:1500 dilution), and anti-β-actin (1:10000 dilution). After washing (x4) in TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:3000 dilution) in blocking buffer for 60 min at room temperature. After further washing (x4) in TBST, immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

**Statistical Analysis:** Results are expressed as mean ± SEM for a series of n experiments. Statistical analyses were performed by paired t-tests or ANOVA with the Tukey multiple comparisons test, as appropriate (Instat Software; GraphPad, San Diego, CA). p values ≤ 0.05 were considered to be significant.
RESULTS

**UDCA exerts antisecretory actions on colonic epithelial cells in vitro.** As previously reported, UDCA (500 µM) did not alter basal I_{sc} across cultured monolayers of T_{84} cells (Figure 1A), although its parent bile acid, CDCA (500 µM) stimulated responses of 40 ± 2.69 µA/cm² within 5 mins. However, UDCA pretreatment significantly attenuated responses to the prototypical Ca^{2+} and cAMP-dependent secretagogues, carbachol (CCh 100 µM) and forskolin (FSK; 10 µM), to 14.0 ± 3.8% and 40.2 ± 7.4% of those in control cells, respectively (n = 18; Figure 1A). The antisecretory effect of UDCA was concentration-dependent, with an EC_{50} of approximately 200 µM, and was rapid-in-onset, occurring within 1 min after its addition (Figure 1B and 1C). Interestingly, UDCA effects on cAMP-dependent responses were slower in onset, taking up to 30 mins to become apparent (Figure 1C). The antisecretory actions of UDCA were reversible and were no longer apparent 120 min after removal of the bile acid (data not shown). Furthermore, UDCA did not alter transepithelial resistance or lactate dehydrogenase release from the cells at any concentration tested (data not shown), suggesting its antisecretory effects are not due to a loss of cell viability.

The effects of UDCA on voltage-clamped human colonic tissue were also examined. After mounting in Ussing chambers, tissues were first treated with tetrodotoxin (TTX) to remove the possible influence of enteric nerves. Similar to our findings in cultured epithelial cells, UDCA alone was without effect on basal I_{sc} (data not shown), but significantly attenuated subsequent agonist-induced responses in a concentration-dependent manner (Figure 1D).

To determine effects of UDCA on electrogenic Na^{+} absorption through ENaC, a previously published approach was employed to induce ENaC expression in T_{84} cells (Iordache and Duszyk, 2007). Pretreatment of cells with 4-phenylbutyrate (4-PB) for 24 hrs induced ENaC protein expression (data not shown) and a basal I_{sc} of 7.7 ± 0.1 µA/cm². While this 4-PB-induced current was abolished by the ENaC blocker, amiloride (10 µM), treatment with UDCA (500 µM) was without effect (Figure 1E).
In vivo administration of UDCA enhances colonic secretory responses in mice. To examine effects of UDCA administration in vivo, mice were injected intraperitoneally with UDCA (25 mg/kg), and after 4 hrs their colons were removed, stripped of smooth muscle, and mounted in Ussing chambers for \( I_{sc} \) measurements. Interestingly, in direct contrast to its antisecretory effects in cultured epithelia, UDCA enhanced secretagogue-induced responses in ex vivo colonic tissues (Figure 2A). We hypothesised that such contrasting effects could be due to bacterial metabolism of UDCA to LCA in vivo. This hypothesis was supported by our findings that colonic LCA levels were significantly increased in UDCA-treated mice from 9.7 ± 1.6 nmol/g (4% of total BA) to 33.4 ± 6.5 nmol/g (16% of total BA) in UDCA-treated mice (Figure 2B). Of the caecal bile acids measured, LCA alone showed significant alterations after treatment with UDCA, and total caecal bile acid levels were similar in control (206 ± 15.5 nmol/g) and UDCA-treated mice (215 ± 18.0 nmol/g). Interestingly, when we tested its effects in Ussing chambers, we found that, in direct contrast to UDCA, LCA significantly potentiated agonist-induced secretory responses in \( T_{84} \) cells (Figure 2C) and human tissue (Figure 2D).

In vivo administration of a metabolically stable UDCA analogue inhibits colonic secretory responses in mice. To further test the hypothesis that prosecretory effects of UDCA in vivo are due to its bacterial metabolism, we employed a non-metabolisable UDCA analogue, 6\( \alpha \)-methyl-UDCA (6\( \alpha \)-MUDCA). Methylation of UDCA in the 6-position sterically hinders bacterial 7-dehydroxylases, thereby preventing its metabolism to LCA (Roda et al., 1995). We confirmed the stability of the compound in mice where, in contrast to UDCA, intraperitoneal administration of 6\( \alpha \)-MUDCA did not alter caecal LCA levels (Figure 3A). Treatment of voltage-clamped \( T_{84} \) cells with 6\( \alpha \)-MUDCA inhibited both CCh- and FSK-stimulated secretory responses to a similar degree as did UDCA (Figure 3B), confirming that it retains the antisecretory actions of UDCA. However, in contrast to our findings with UDCA, in vivo administration of 6\( \alpha \)-MUDCA significantly attenuated agonist-induced secretory responses in ex vivo colonic tissues (Figure 3C). Interestingly, 6-MUDCA did not alter SGLT-1 activity in ex vivo mouse...
jejunal tissues, suggesting the compound specifically inhibits epithelial secretory function (Figure 3D).

**Sidedness of antisecretory actions of UDCA.** To further characterise antisecretory actions of UDCA on colonic epithelial cells, we examined the sidedness of responses to the bile acid. Basolateral addition of UDCA was more effective in inhibiting agonist-induced secretory responses than was apical addition (Figure 4A). Interestingly however, the taurine-conjugated derivative of UDCA (TUDCA; 500 μM), which is unable to cross cell membranes unless a transporter is present, was ineffective when added apically, but was equipotent to UDCA when added basolaterally (Figure 4B). We also examined the sidedness of UDCA effects on agonist-induced secretory responses across sections of human colon in Ussing chambers. Similar to its effects in cultured T84 cells, UDCA was more effective when applied basolaterally, with secretory responses to CCh being inhibited to 40.4 ± 8.1% of controls compared to 69.3 ± 14.1% inhibition after apical addition (n = 4; p < 0.05).

**UDCA inhibits Na+/K+ ATPase pump activity and K+ channel currents in colonic epithelial cells.** To begin to elucidate how UDCA exerts its antisecretory effects, we analyzed the effects of the bile acid on the activity of several transport proteins that comprise the Cl− secretory pathway. Under conditions that specifically isolate basolateral Na+/K+ ATPase pump activity or K+ conductances, UDCA (500 μM) significantly inhibited CCh (100 μM)-stimulated I_sc responses to 16.2 ± 3.9% and 13.7 ± 4.1% of controls, respectively (Figures 5A–B). In contrast, UDCA did not alter FSK-stimulated apical Cl− currents which were 100.9 ± 22.2% of control responses (Figure 5C).

Finally, to determine if UDCA has its antisecretory effects by altering the surface expression of Na+/K+ ATPase pumps and K+ channels at the cell surface, we employed a cell surface biotinylation approach to quantify expression of these proteins in the basolateral membrane. However, these experiments revealed that there were no significant alterations in surface expression of either the Na+/K+ ATPase α subunit or KCNN4 in UDCA-treated cells (Figures 5D and 5E).
DISCUSSION

It is well-established that dihydroxy bile acids stimulate intestinal fluid and electrolyte secretion and that their increased colonic delivery in conditions of bile acid malabsorption causes diarrhoea (Chadwick et al., 1979; Keely et al., 2007). However, a notable exception is UDCA, which based on its lack of secretory actions in guinea pig, rabbit, and canine colon in vitro and in vivo, has been previously characterised as a “non-secretory” bile acid (Mekjian et al., 1971; Rahban et al., 1980; Gelbmann et al., 1995). Here, we present new data to show that in addition to being non-secretory, UDCA also exerts potent antisecretory effects on colonic epithelial cells. The actions of UDCA were observed both in cultured cells and in pharmacologically-denervated human colonic tissue, suggesting they are due to a direct action of the bile acid on the epithelial cells. It was interesting to note that the time course by which UDCA has its antisecretory actions depends on the particular type of secretagogue in question, with inhibition of Ca\(^{2+}\)-dependent responses occurring more rapidly than that of cAMP-dependent responses. This suggests that different mechanisms might underlie the antisecretory effects of UDCA on Ca\(^{2+}\) and cAMP-dependent agonists. Although the concentrations at which UDCA has its antisecretory actions (50 µM–1 mM) might not normally occur in vivo (De Kok et al., 1999), during conditions of bile acid malabsorption or when it is used therapeutically, levels of the bile acid increase significantly in the colonic lumen and serum (van Gorkom et al., 2002; Hess et al., 2004).

Intriguingly, we found that, in contrast to its antisecretory actions in vitro, intraperitoneal injection of UDCA to mice potentiated secretagogue-induced responses across ex vivo colonic tissues. Such prosecretory effects of UDCA when administered in vivo may provide new insights into the diarrhoeal side effects that can occur when the bile acid is used therapeutically (Alberts et al., 2005). While, the underlying cause of this diarrhoeal effect is not known, it has been hypothesised to be due to bacterially-mediated re-epimerisation of the 7 beta-OH group of UDCA to give CDCA, which is prosecretory (Hempfling et al., 2003). However, UDCA is preferentially metabolised in vivo to the monohydroxy bile acid, LCA, which has been shown to be the predominant colonic bile
acid in humans treated with UDCA (van Gorkom et al., 2002). We also found caecal LCA levels to increase dramatically upon administration of UDCA to mice. Although known to be a hepatotoxic bile acid, the effects of LCA on colonic transport function are poorly defined. Here, we found that LCA potentiates epithelial responses to secretagogues and it therefore may act to oppose the antisecretory actions of UDCA. Thus, we hypothesised that metabolism to LCA might underlie the prosecretory effects of UDCA in vivo, and further that derivatives of UDCA, which cannot be metabolised by 7-dehydroxylation, should have antisecretory actions in vivo. To test this hypothesis we used 6α-MUDCA, a 6-methylated derivative of UDCA that is completely resistant to bacterial dehydroxylation (Roda et al., 1995), but which retains the antisecretory effects of UDCA in vitro. Upon administration to mice, 6α-MUDCA did not alter caecal LCA levels and significantly inhibited secretagogue-induced responses across voltage-clamped colonic tissues. Thus, our data support the hypothesis that it is because of its metabolism to LCA that UDCA enhances colonic secretion in vivo, and that this effect may underlie the diarrhoeal side effects that occur when UDCA is used therapeutically for liver disease. Importantly, our data also suggest that non-metabolisable analogues of UDCA may be good candidates for development into a new class of antidiarrhoeal drug.

Although significant inhibition of agonist-induced responses was observed upon apical addition of UDCA, the bile acid was found to be more effective when added basolaterally. A similar pattern of responses to apical and basolateral UDCA was observed in voltage-clamped human colonic tissues. Interestingly, the taurine conjugate of UDCA, TUDCA, was only effective when applied basolaterally to T84 monolayers. TUDCA is more hydrophilic than UDCA, and unlike UDCA it cannot enter cells by passive diffusion across the lipid membrane. However, bile acid transporters are expressed on the basolateral membrane of colonic epithelia (Weihrauch et al., 2006), which likely explains the increased efficacy of both TUDCA and UDCA when applied basolaterally. These data are consistent with our previous findings which show that conjugated bile acids do not cross the apical membrane of T84 cells and that uptake of UDCA occurs more rapidly across the basolateral, than apical membrane (Keely et al., 2007). Importantly, these data also suggest that UDCA, and its derivatives, must enter the
cells before they can exert their antisecretory actions. Furthermore, conditions which disrupt tight junction integrity would be expected to result in enhanced epithelial delivery of UDCA or its conjugates to the basolateral membrane, thereby increasing their biological actions.

Using well-established approaches to specifically measure Na\(^+\)/K\(^+\) ATPase activity, K\(^+\) channel currents, and Cl\(^-\) channel currents, we found that UDCA significantly inhibited both Na\(^+\)/K\(^+\) ATPase activity and Ca\(^{2+}\)-activated K\(^+\) channel currents, but not FSK-stimulated apical Cl\(^-\) channel currents. Thus, upon entry into colonic epithelial cells, UDCA rapidly inhibits key transport proteins required for Cl\(^-\) secretion to occur. We hypothesised that such effects of UDCA could be mediated by altered trafficking of transport proteins to the cell surface, and therefore carried out cell surface biotinylation analyses. However, we detected no reductions in the surface localisation of either Na\(^+\)/K\(^+\) ATPase pumps or K\(^+\) channels after UDCA treatment, suggesting that the antisecretory effects of the bile acid are not mediated by altered trafficking of transport proteins to the cell surface. Thus, how UDCA inhibits the activity of Na\(^+\)/K\(^+\) ATPase pumps and K\(^+\) channels remains to be determined but several possibilities exist. For example, UDCA may alter the generation of signals that activate these transport proteins or alternatively could alter their association with regulatory proteins. Several proteins are known to bind to and regulate Na\(^+\)/K\(^+\) ATPase activity, including FXYD proteins (Garty & Karlish, 2006), translationally-controlled tumor protein (Jung et al., 2004), and MONaKA (modulator of Na\(^+\)/K\(^+\) ATPase) (Mao et al., 2005). Indeed, such regulatory proteins provide an attractive mechanism for inhibition of the Na\(^+\)/K\(^+\) ATPase pump, since many are expressed in a cell-specific manner and could explain why UDCA specifically inhibits Cl\(^-\) secretory responses, while Na\(^+\) absorptive processes, including electrogenic Na\(^+\) absorption through ENaC and SGLT-1 activity, remain unaltered. Similarly, proteins that regulate the activity of KCNN4, the Ca\(^{2+}\)-dependent K\(^+\) channel, are known to exist and could represent targets for UDCA action (Joiner et al., 2001). Future studies will focus on elucidating the molecular mechanisms by which UDCA inhibits the activities of these transport proteins and also to characterise the effects of UDCA on Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter activity, which is also a key component of the epithelial secretory pathway.
In summary, UDCA is an antisecretory bile acid that has its effects by inhibition of multiple components of the Cl⁻ secretory pathway. However, antisecretory actions of UDCA are not likely to be observed under normal conditions *in vivo* due to its rapid metabolism by colonic bacteria to LCA, which in contrast to UDCA, enhances epithelial secretory function. Thus, our data provide new insights that may explain the occurrence of diarrhoea in patients on UDCA therapy, and also suggest that metabolically stable analogues of the bile acid may be good candidates for development of a new class of antidiarrhoal drug. By virtue of their direct actions on epithelial secretory function, along with the well-established safety profile UDCA in humans, such drugs could have distinct advantages over currently available antidiarrhoeal therapies.
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AUTHOR CONTRIBUTIONS

OK: study design/data acquisition/analysis/interpretation, manuscript preparation; MM, JW, CC, MS: data acquisition/analysis/interpretation/ manuscript preparation; AR, RP: development of 6-MUDCA, data acquisition/interpretation; PF, FM, JG: study concept and design; AH: study concept and design, manuscript preparation; SK: obtained funding, study concept and design, manuscript preparation.

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TRANSLATIONAL PERSPECTIVES

These current studies reveal new roles for the colonic bile acids, ursodeoxycholic acid (UDCA) and lithocholic acid (LCA), in regulating epithelial secretory function. While UDCA exerts direct antisecretory effects on colonic epithelial cells, LCA has contrasting actions that enhance agonist-induced secretion. Since UDCA is rapidly metabolised in vivo by colonic bacteria to LCA, our data provide a new explanation as to why UDCA can cause diarrhoea when it is used in clinical settings. Furthermore, our data suggest that UDCA analogues that cannot be metabolised by colonic bacteria may be of therapeutic use in treating intestinal disorders associated with secretory diarrhoea. However, when considering the translational perspectives of this study, it is important to note that the effects of UDCA and LCA described here occur at relatively high concentrations that are not likely to occur under normal circumstances in vivo. Thus, these effects should be considered as pharmacological, rather than physiological, and are likely to be applicable only to situations where UDCA is administered therapeutically. Further studies are now required to translate our findings into animal models of intestinal disease to determine if metabolically stable UDCA analogues may ultimately be useful in treating secretory diarrhoea in humans. Finally, our data should also be considered in a wider context where, as a consequence of its well-established cytoprotective actions, UDCA is under investigation for a range of intestinal and extra-intestinal pathological conditions, including cancer, neurological diseases and atherolsclerosis. In such settings, the potential benefits of an absorbable, non-metabolisable analogue, which retains the therapeutic activity of UDCA are clear.
FIGURE LEGENDS

Figure 1: UDCA inhibits colonic epithelial secretory responses in vitro. A) T84 cell monolayers were mounted in Ussing chambers and treated bilaterally with ursodeoxycholic acid (UDCA; 500 µM) for 15 min, before Cl⁻ secretory responses to basolateral carbachol (CCh; 100 µM) and apical forskolin (FSK; 10 µM) were measured as changes in short circuit current (ΔIsc) (n = 18). B) T84 cells were exposed to bilateral UDCA at differing concentrations (50 µM–1 mM) for 15 min and subsequent Isc responses to CCh and FSK were measured (n = 6–18). C) UDCA (500 µM) was added bilaterally to voltage-clamped monolayers of T84 cells for various periods of time before measuring secretory responses to CCh and FSK (n = 4). D) Muscle-stripped sections of human colon were mounted in Ussing chambers and monitored for changes in Isc. The basolateral solution contained tetrodotoxin (TTX; 1 µM) to remove the influence of enteric nerves. After stabilisation of the Isc, tissues were treated bilaterally with UDCA at various concentrations for 15 mins prior to stimulation with CCh and FSK (n = 8). E) T84 cells were pretreated with 4-phenylbutyrate (5 mM; 24 h) to induce amiloride-sensitive Na⁺ channel (ENaC) activity before mounting in Ussing chambers. Cells were then sequentially treated with UDCA (500 µM) and amiloride (10 µM) and changes in Isc were noted (n = 8). * p < 0.05; ** p < 0.01; *** p < 0.001. n.d. denotes “not determined”.

Figure 2: UDCA administration in vivo potentiates colonic epithelial secretory responses. A) Male C57/BL6 mice were intraperitoneally injected with UDCA (25 mg/kg), or phosphate buffered saline (PBS) vehicle, and were then sacrificed after 4 h. Muscle-stripped sections of colonic mucosa were mounted in Ussing chambers and changes in CCh (100 µM) and FSK (10 µM)-induced Isc were measured (n = 5). The inset shows peak changes in Isc induced by CCh and FSK in UDCA-treated mice expressed as a % of control responses. B) The caecal contents from control and UDCA-treated mice were collected and LCA levels were measured by HPLC mass spectrometry (n = 4). C) Monolayers of T84 cells were mounted in Ussing chambers and were treated bilaterally with LCA (50–250 µM) for 15 min before measuring Isc responses to CCh (100 µM) (n = 9). D) Muscle-stripped sections of human colon were mounted in Ussing chambers in the
presence of basolateral TTX (1 µM) to remove the influence of enteric nerves. After stabilisation of the $I_{sc}$, tissues were treated bilaterally with LCA (50 µM) for 15 min prior to stimulation with basolateral CCh (100 µM) ($n = 5$). * $p < 0.05$; *** $p < 0.001$.

**Figure 3: 6α-methyl UDCA inhibits colonic epithelial Cl$^-$ secretion in vitro and in vivo.** A) Male C57/BL6 mice were intraperitoneally injected with 6-α-MUDCA (25 mg/kg), or PBS vehicle, and were then sacrificed after 4 h. The caecal contents from control and 6α-MUDCA-treated mice were collected and LCA levels were measured by mass spectrometry ($n = 4$). B) Voltage-clamped monolayers of T$\text{84}$ cells were pretreated with UDCA or 6α-MUDCA (100–500 µM) for 15 min before measuring CCh (100 µM) or FSK (10 µM)-stimulated $I_{sc}$ responses. Maximal changes in $I_{sc}$ in response to CCh and FSK were expressed as % of responses in controls ($n = 7$). C) Mice were injected intraperitoneally with 6α-MUDCA (25 mg/kg) and after 4 h muscle-stripped sections of colonic mucosa were mounted in Ussing chambers and changes in $I_{sc}$ in response to CCh and FSK were measured. The inset shows peak changes in $I_{sc}$ induced by CCh and FSK in 6α-MUDCA-treated mice expressed as a % of control responses ($n = 5$). D) Sections of jejunum from control or 6α-MUDCA-treated mice were mounted in Ussing chambers for measurements of SGLT-1 activity. After a 20 min equilibration period, glucose (25 mM) was added apically and mannitol (25 mM) basolaterally and subsequent changes in $I_{sc}$ were measured ($n = 6$) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

**Figure 4: Sidedness of the antisecretory effects of UDCA and TUDCA on colonic epithelial cells.** A) T$\text{84}$ cell monolayers were mounted in Ussing chambers and treated apically, basolaterally, or bilaterally with UDCA (500 µM) for 15 min, before Cl$^-$ secretory responses to basolateral CCh (100 µM) or FSK (10 µM) were measured as changes in $I_{sc}$ ($n = 13$). B) Voltage-clamped T$\text{84}$ cell monolayers were treated apically, basolaterally, or bilaterally with tauro-UDCA (TUDCA; 500 µM) for 15 min, before Cl$^-$ secretory responses to basolateral CCh (100 µM) or FSK (10 µM) were measured ($n = 10$) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 
Figure 5: UDCA inhibits Na\(^+\)/K\(^+\) ATPase pump activity and basolateral K\(^+\) currents in colonic epithelial cells. Monolayers of T\(_{84}\) cells were mounted in Ussing chambers for measurements of A) Na\(^+\)/K\(^+\)-ATPase activity (n = 7), B) basolateral K\(^+\) currents (I\(_{\text{K}^+}\); n = 5 or C) apical Cl\(^-\) currents (I\(_{\text{Cl}^-}\); n = 10) as described in Methods. The insets to each panel show maximal changes in I\(_{\text{sc}}\) (ΔI\(_{\text{sc}}\)) in response to agonist stimulation under each experimental condition expressed as a % of control responses. D) After treatment with UDCA (500 µM; 15 min) total cellular and basolateral surface expression of the Na\(^+\)/K\(^+\)-ATPase α\(_1\) subunit was analysed by western blotting or cell surface biotinylation, respectively. The lower panel shows densitometric analysis of 8 similar experiments. E) After treatment with UDCA (500 µM; 15 min) total cellular and basolateral surface expression of KCNN4 was analysed. The lower panel shows densitometric analysis of 3 similar experiments.
Figure 1

A

B

C

D

E
Figure 3

A

B

C

D