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Investigating the effects of physiological bile acids on GLP-1 secretion and glucose tolerance in normal and GLP-1R$^{-/-}$ mice

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Abstract

Physiological secretion of bile acids has previously been linked to the regulation of blood glucose. GLP-1 is an intestinal peptide hormone with important glucose-lowering actions, such as stimulation of insulin secretion and inhibition of glucagon secretion. In this investigation, we assessed the ability of several bile acid compounds to secrete GLP-1 *in vitro* in STC-1 cells. Bile acids stimulated GLP-1 secretion from 3.3- to 6.2-fold but some were associated with cytolytic effects. Glycocholic and taurocholic acids were selected for *in vivo* studies in normal and GLP-1R$^{-/-}$ mice. Oral glucose tolerance tests revealed that glycocholic acid did not affect glucose excursions. However, taurocholic acid reduced glucose excursions by 40% in normal mice and by 27% in GLP-1R$^{-/-}$ mice, and plasma GLP-1 concentrations were significantly elevated 30 min post-gavage. Additional studies used incretin receptor antagonists to probe involvement of GLP-1 and GIP in taurocholic acid-induced glucose lowering. The findings suggest that bile acids partially aid glucose regulation by physiologically enhancing nutrient-induced GLP-1 secretion. However, GLP-1 secretion appears to be only part of the glucose-lowering mechanism and our studies indicate that the other major incretin GIP is not involved.

Keywords: bile acids; endocrine; GLP-1; glucose; gut hormones; incretin; intestine; secretion.

Introduction

Bile acids are a family of steroid molecules which play important physiological roles in the absorption of dietary lipids, the solubilisation of lipid-soluble vitamins and the regulation of cholesterol biosynthesis (Russell, 2003). However, the actual role of bile acids appears more complex and unexpectedly bile acids appear to act as potent intra-intestinal hormones (Vallim and Edwards, 2009). Initial studies found that specific bile acids bound and activated the nuclear receptor FXR to affect changes in cholesterol metabolism (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999) and glucose metabolism (Ma et al., 2006; Zhang et al., 2006). Plaisancie and colleagues found that bile acids stimulate release of glucagon-like peptide-1 (GLP-1) in isolated vascularly perfused rat colon (Plaisancie et al., 1995). More recently a G-protein-coupled receptor called TGR5 has been identified which acts as a cell-surface receptor for bile acids (Maruyama et al., 2002; Kawamata et al., 2003; Sato et al., 2007). Interestingly, it appears that bile acids act through TGR5 to promote GLP-1 secretion in the murine enteroendocrine cell line, STC-1 (Katsuma et al., 2005). Bile acids such as lithocholic acid and deoxycholic acid also appear to dose-dependently stimulate intracellular cAMP production in STC-1 cells (Katsuma et al., 2005). Furthermore, specific inhibition of adenylate cyclase significantly suppresses bile acid-induced GLP-1 secretion, suggesting that bile acids induce GLP-1 secretion via the production of intracellular cAMP in STC-1 cells (Katsuma et al., 2005).

The actions of the incretin GLP-1 contribute significantly to the enteroinsular axis, which is a network of neural and endocrine signals connecting the intestine and the pancreas potentiating nutrient-induced insulin secretion (Creuzfeldt, 1979). Incretins are peptides released from specialised enteroendocrine cells in the intestinal lining. Cells monitor the intraluminal contents of the intestine and following appropriate stimulation they release intracellular secretory granules on the basolateral surface, and from here the endocrine hormones can enter capillaries of the blood circulation (Solcia et al., 1998). Two incretin hormones which potently stimulate insulin secretion at physiological concentrations: GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) (Baggio and Drucker, 2007). GLP-1 is a product of gut L-cells located in the distal small intestine, whereas GIP is produced and secreted by gut K-cells located in the proximal small intestine (Baggio and Drucker, 2007). STC-1 cells are derived from an endocrine tumour of mouse intestine (Rindi et al., 1990) and their secretory responses have previously been studied (Katsuma et al., 2005; Okawa et al., 2009; Hand et al., 2010; Geraedts et al., 2011). An early report concluded that approximately 7% of this heterogeneous population of cells also produce detectable levels of GIP (Rindi et al., 1990). Furthermore, overexpression of the GIP promoter in

STC-1 cells (pGIP/Neo) produces a cell line which mimics many of the characteristics of a K-cell GIP-secreting cell line (Ramshur et al., 2002).

In this study, we examined the relative *in vitro* potency of six physiological bile acid compounds (Figure 1) to stimulate GLP-1 secretion. We found that amino acid-derivatised bile acids, glycocholic and taurocholic acids were the most potent GLP-1 secretagogues *in vitro* and these compounds were selected for studies in normal and GLP-1R$^{-/-}$ mice. The aim of *in vivo* studies was to establish whether bile acids could bring about glucose-lowering in mice and to examine the involvement of GLP-1 secretion in this effect. Our findings reveal that taurocholic acid has a potent antihyperglycaemic action which is partly mediated via secretion of the incretin hormone GLP-1; however, other glucoregulatory mechanisms appear to be involved.

Results

Effects of bile acids on murine STC-1 cells

Figure 2A shows the GLP-1 secretory responses of bile acids after 3 h incubation period. Remarkably, all bile acid incubations increased GLP-1 concentrations in the test buffer. Lithocholic, cholic, chenodeoxycholic and deoxycholic acids increased GLP-1 secretion (3.3-, 3.8-, 4.2-fold and 4.2-fold, respectively; $p<0.05$ to $p<0.001$). The most potent GLP-1 secretagogues were taurocholic acid (6.1-fold; $p<0.01$) and glycocholic acid (6.2-fold; $p<0.01$) and as such were selected for acute *in vivo* studies. Lithocholic acid and glycocholic acid showed appreciable cytolytic activity against STC-1 cells as indicated by elevations in extra cellular levels of the cytosolic enzyme, lactate dehydrogenase (LDH) (Figure 2B). Lithocholic acid and glycocholic acid increased LDH release by 23±3% and 14±3%, respectively ($p<0.05$). Deoxycholic, cholic, chenodeoxycholic and taurocholic acids did not affect LDH release.

Acute glucose-lowering effects of bile acids in normal and GLP-1R$^{-/-}$ mice

Glycocholic acid did not significantly lower glycaemic excursions in either normal (Figure 3A) or *GLP-1R*$^{-/-}$ mice (Figure 3B). Normal and GLP-1R$^{-/-}$ mice receiving glucose and taurocholic acid had significantly lower responses compared with mice receiving glucose alone. Analysis by area under the curve (ΔAUC0–105) revealed that taurocholic acid reduced responses in normal mice by 40% ($p<0.01$; Figure 3A). Taurocholic acid retained antihyperglycaemic activity in GLP-1R$^{-/-}$ mice; however, AUC values were reduced by 27% compared with glucose alone ($p<0.05$; Figure 3B).

A second series of experiments was conducted to assess the effect of incretin receptor antagonism on taurocholic acid-induced glucose lowering. Normal mice receiving oral taurocholic acid had lower glucose excursions than those receiving glucose alone (38% lower; $p<0.01$; Figure 4A) and this was not affected by the administration of intraperitoneal saline. However, excursions in mice receiving taurocholic acid in combination with exendin(9–39) were significantly higher than mice receiving taurocholic acid and saline (31% higher; $p<0.05$; Figure 4A). GLP-1R$^{-/-}$ mice receiving oral taurocholic acid had lower glucose excursions than those receiving glucose alone (30% lower; $p<0.05$; Figure 4B) and this was unaffected by the administration of intraperitoneal saline. Responses in GLP-1R$^{-/-}$ mice receiving taurocholic acid in combination with intraperitoneal (Pro^3)GIP did not differ significantly from mice receiving taurocholic acid and saline. Taurocholic acid administration led to elevated plas-

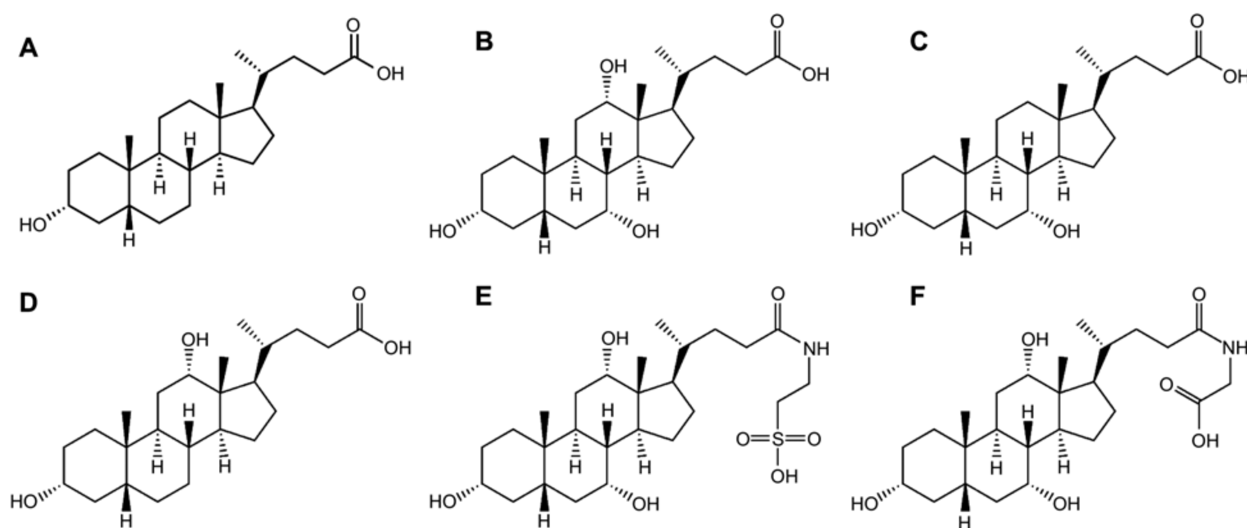

Figure 1 Molecular structures of physiological bile acids.
Bile acids are a family of steroid molecules with four rings and a five or eight carbon side chain terminating in a carboxylic acid group. Shown are the structures of (A) lithocholic acid, (B) cholic acid, (C) chenodeoxycholic acid, (D) deoxycholic acid, (E) taurocholic acid and (F) glycocholic acid.

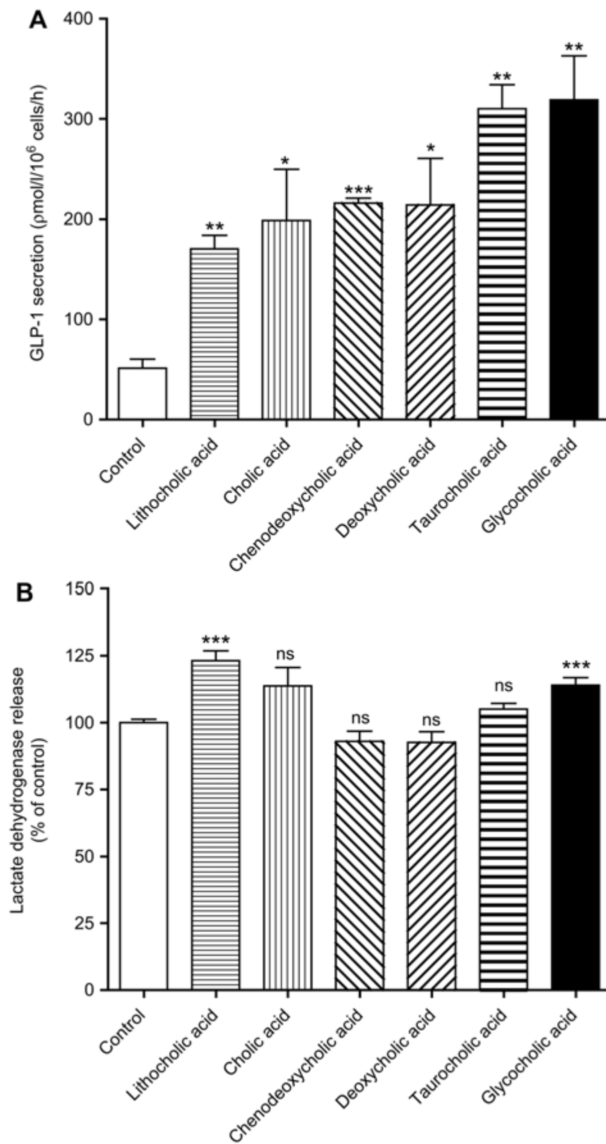

Figure 2 Effects of bile acids on GLP-1 secretion and LDH release from STC-1 cells.

STC-1 cells were incubated for 3 h with lithocholic, cholic, chenodeoxycholic, deoxycholic, taurocholic or glycocholic acids (100 μM) before determination of GLP-1 secretion (A) and LDH release (B). Results are mean±SEM (n=8). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with vehicle control; ns, not significant.

ma GLP-1 concentrations 30 min post-gavage in normal (109% higher; $p<0.05$; Figure 5A) and GLP-1R$^{-/-}$ mice (73% higher; $p<0.05$; Figure 5B).

GIP secretion in pGIP/Neo STC-1 cells

Incubation of *pGIP/Neo* cells with taurocholic acid (100 μM) did not elicit any significant GIP response (Figure 6). However, glycocholic acid (100 μM) significantly increased GIP secretion by 4.4-fold compared with vehicle control (Figure 6; $p<0.05$).

Discussion

There have been several recent reports linking bile acids to physiological glucose regulation (Han et al., 2004; Watanabe et al., 2006; Sato et al., 2007; Fonseca et al., 2008; Goldberg et al., 2008; Shaham et al., 2008; Roberts et al., 2011). Postprandial increases in bile acid concentrations have been reported in human volunteers after ingestion of a standard liquid meal (De Barros et al., 1982). Another study which simultaneously measured 191 blood metabolites in human volunteers ingesting glucose noted significant increases in plasma bile acid concentrations (Shaham et al., 2008). The concentration of bile acids (glycocholic, glycochenodeoxycholic and taurochenodeoxycholic acid) doubled within 30 min of glucose consumption and remained elevated for a period of up to 2 h (Shaham et al., 2008). Furthermore, individuals with impaired glucose tolerance and high fasting insulin levels exhibited a blunted excursion of glycochenodeoxycholic acid (Shaham et al., 2008). Postprandial bile acid responses in humans appear to correlate tightly with plasma GLP-1 levels (Roberts et al., 2011). Feeding high-fat fed mice a bile acid-enriched diet for 9 weeks prevented the onset of hyperglycaemia, obesity and insulin resistance (Ikemoto et al., 1997). A natural compound from olive leaves called oleanoic acid, which has a similar molecular structure to bile acids, lowers blood glucose and insulin levels in high-fat fed mice and enhances glucose tolerance (Sato et al., 2007). Furthermore, clinical trials in type 2 diabetic patients clearly demonstrated that bile acid sequestrant drugs (which prevent bile acid reabsorption by the gut) improve glycaemic control (Garg and Grundy, 1994; Fonseca et al., 2008; Goldberg et al., 2008).

Following contraction of the gall bladder bile is ejected directly into the lumen of the intestine and the bile acids are brought into contact with chyme and the intestinal lining. Several recent studies have proposed that bile acids stimulate the release of GLP-1 from intestinal enteroendocrine cells, and it has been postulated that bile acids act as endogenous GLP-1 secretagogues which possibly influence postprandial glucose metabolism. Here, we investigated the effect of various bile acids on GLP-1 secretion from STC-1 cells and established that taurocholic and glycocholic acids were among the most potent secretagogues. The GLP-1 secretory activity of bile acids has been previously reported. Plaisancie et al. (1995) found that taurocholic, cholic, deoxycholic and hyodeoxycholic acids increased GLP-1 secretion in perfused rat colon. Katsuma et al. (2005) reported that lithocholic and deoxycholic acids increased GLP-1 secretion in STC-1 cells. Our findings are in broad agreement with these studies; however, we also provide evidence that some GLP-1 secretion induced by some bile acids could relate to possible cytolytic affects. GLP-1 secretion induced by cholic, chenodeoxycholic, deoxycholic and taurocholic acids were not associated with LDH release and therefore the integrity of the plasma membrane does not appear to be affected. By contrast, lithocholic and glycocholic acids led to elevated LDH release and it is possible that some of the observed GLP-1 secretion is due to cytolysis.

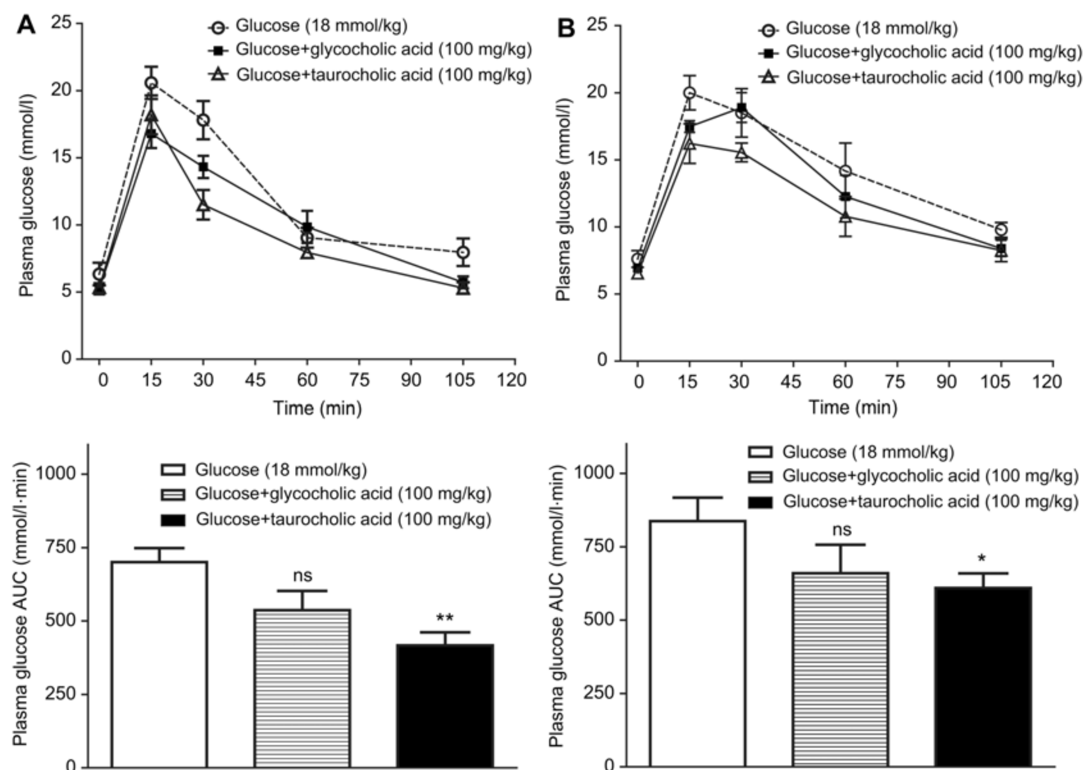

Figure 3 Effects of oral glycocholic and taurocholic acids on glucose tolerance in normal and GLP-1R$^{-/-}$ mice.
Taurocholic acid (100 mg/kg) or glycocholic acid (100 mg/kg) were administered with glucose (18 mmol/kg) to normal (A) and *GLP-1R*$^{-/-}$ mice (B), and glucose responses were monitored at 0, 15, 30, 60 and 105 min. Results are mean±SEM (n=6). *$p<0.05$ and **$p<0.01$ compared with glucose alone; ns, not significant.

Given that TGR5 is more sensitive to lithocholic and deoxycholic acids than chenodeoxycholic acid it is surprising that taurocholic and glycocholic acids were more potent GLP-1 secretagogues than lithocholic acid; however, the affinity and activity of these bile acids for the TGR5 receptor has not yet been investigated (Kawamata et al., 2003). It could be speculated that derivatising bile acids with glycine or taurine improves GLP-1 secretion by altering their affinity/activation for TGR5. However, it is also possible that bile acids such as taurocholic acid interact with other unelucidated targets.

As the most potent GLP-1 secretagogues, taurocholic and glycocholic acids were initially selected for *in vivo* studies. In glucose tolerance tests glycocholic acid did not alter glycaemic responses. Taurocholic acid possessed glucose-lowering activity in normal mice and this activity was also evident in GLP-1R$^{-/-}$ mice; however, the effect here was less pronounced. Similar differences in glucose responses were also observed when the GLP-1 receptor antagonist, exendin(9–39) was employed. These findings indicated that GLP-1 secretion was likely to be involved in glucose-lowering, but it also indicated that other glucoregulatory mechanisms were involved. We hypothesised that bile acids might also stimulate secretion of the other major incretin GIP and attempted to ascertain this by employing a specific GIP receptor antagonist (Pro^3)GIP (Irwin et al., 2006; Parker et al., 2007) in GLP-1R$^{-/-}$ mice. The results did not support a role for GIP in taurocholic acid-induced glucose lowering. Finally, we investigated GIP secretion *in vitro* and this demonstrated that taurocholic acid did not stimulate GIP secretion. Strangely, glycocholic acid caused significant GIP secretion and it is uncertain whether this is a specific secretory mechanism or whether it relates to the observed cytolytic activity.

Some observations in the literature indicate that bile acid-related compounds have glucose-lowering actions. Incorporating oleanoic acid into the diet of high-fat fed mice significantly lowers blood glucose levels after only 7 days (Sato et al., 2007). Similarly, adding sodium cholate to the diet of these mice improves glucose tolerance (Ikemoto et al., 1997). Here, we observed an acute glucose-lowering effect induced by a relatively high dose of bile acid. The concentrations administered here (100 mg/kg) are not likely to be achieved physiologically; however, it could indicate a potential role for bile acids in potentiating the enteroinsular axis.

Bile acids are thought to mediate their actions in several ways. Initially a nuclear receptor for bile acids called FXR was discovered, and subsequently three other nuclear receptors (called VDR, PXR and CAR) were uncovered (Vallim and Edwards, 2009). These nuclear receptors are not thought to be responsible for the glucoregulatory effects of bile acids (Maruyama et al., 2002; Kawamata et al., 2003; Sato et al., 2007; Thomas et al., 2008, 2009; Poupon, 2010). Instead a G-protein-coupled receptor called TGR5 is believed to bind

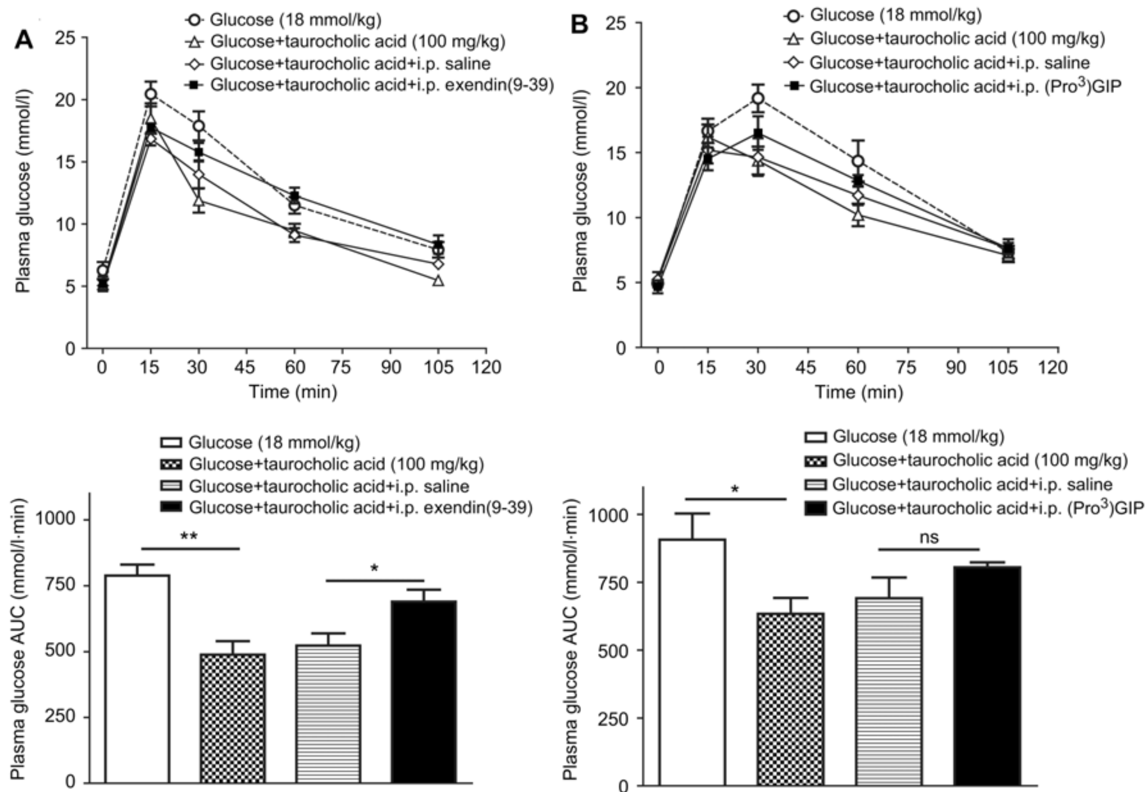

Figure 4 Effects of incretin receptor antagonists on the anti-hyperglycaemic actions of taurocholic acid in normal and GLP-1R$^{-/-}$ mice. Immediately after oral gavage with glucose and taurocholic acid normal mice were given intraperitoneal saline or exendin(9–39) (50 nmol/kg). Similarly, GLP-1R$^{-/-}$ mice were given intraperitoneal saline or (Pro^3)GIP (50 nmol/kg). Blood glucose was analysed immediately before administration and 15, 30, 60 and 105 min post-administration. Results are mean±SEM (n=6). *p<0.05 and **p<0.01 compared as indicated by lines; ns, not significant.

various bile acid compounds on the cell surface and activation of this receptor activates adenlyate cyclase stimulating production of cAMP second messengers which in turn causes GLP-1 secretion from STC-1 cells (Katsuma et al., 2005). TGR5 is highly expressed in the gall bladder, jejunum, ileum and colon (Thomas et al., 2008). Furthermore, genetic gain- and loss-of-function studies underline the importance of the TGR5 signalling pathway in regulating intestinal GLP-1 secretion *in vivo* (Thomas et al., 2009). It has been postulated that specific TGR5 agonists such as 6α-ethyl-23(*S*)-methylcholic acid (INT-777) could provide future therapies for the treatment of type 2 diabetes. The potential efficacy of such an approach is encouraged by the fact that incretin-related therapies are already in clinical use. For example, there are drugs which mimic incretin actions (e.g., byetta, liraglutide) (Green and Flatt, 2007) and drugs which enhance/prolong incretin hormone activity (known as DPP-4 inhibitors or gliptins; e.g., vildagliptin, sitagliptin) (Flatt et al., 2008, 2009). A third approach is now gaining momentum: the possibility of developing incretin secretagogue drugs which enhance secretion of the incretin hormones.

The *in vitro* and *in vivo* findings in this study suggest that bile acids partially aid glucose regulation by physiologically enhancing nutrient-induced GLP-1 secretion. However, GLP-1 secretion only appears to be part of the glucose-lowering mechanism and our studies indicate that the other major incretin GIP is not involved. Other possible antidiabetic mechanisms of bile acids have been reported. Bile acids can enter the liver via the portal vein and may assist the glucoregulatory actions of insulin (Han et al., 2004). Deoxycholic acid and taurocholic acid appear to activate glycogen synthase in rat hepatocytes via a PI3 kinase/AKT/GSK pathway (Han et al., 2004). Furthermore, bile acids seem to activate insulin receptors and enhance phosphorylation of insulin receptor substrate-1 and therefore could enhance insulin action (Han et al., 2004). Also, bile acids appear to increase energy expenditure in brown adipose tissue by the activation of an enzyme called type 2 iodothyronine deiodinase which leads to activation of thyroid hormone (Watanabe et al., 2006). There is a growing body of evidence which suggests that bile acids are important physiological mediators of glucose homeostasis and this warrants further investigation.

Materials and methods

Reagents

A polyclonal primary antibody [raised against GLP-1(7–36)amide] was provided by the regional Regulatory Peptide Laboratory, Royal Victoria Hospital, Belfast. GLP-1(7–36)amide was purchased from

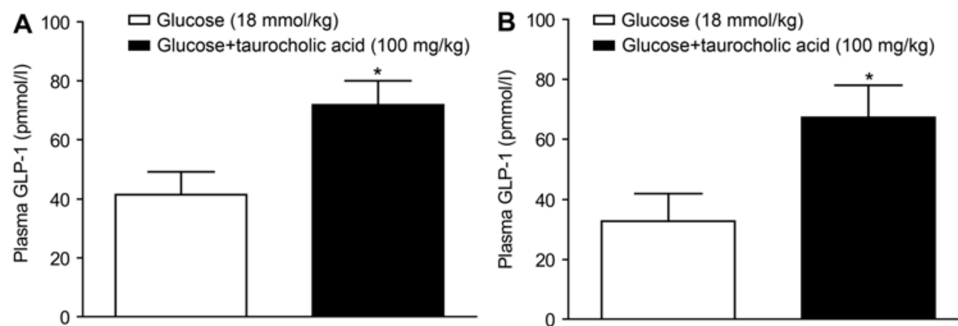

Figure 5 Plasma GLP-1 concentrations in normal mice and GLP-1R$^{-/-}$ mice.
Mice were orally gavaged with glucose (18 mmol/kg) or glucose in combination with taurocholic acid (100 mg/kg) and after 30 min mice were sacrificed and blood samples obtained by cardiac puncture. Samples are mean±SEM (n=6). *$p<0.05$ compared with glucose alone.

EZBiolab (Carmel, IN, USA). Radioiodinated GLP-1 was obtained from Perkin Elmer (Waltham, MA, USA). (Pro^3)GIP was kindly provided by Dr. V. Gault (University of Ulster, Coleraine, UK). Microtitre plates used were Nunc 12-well or 96-well maxisorp/cell culture plates. Lithocholic, cholic, chenodeoxycholic, deoxycholic, taurocholic and glycocholic acids were purchased from Sigma (Poole, Dorset, UK). Bile acid solutions were prepared in prewarmed buffer immediately prior to experiments and a sonicating water bath was used as necessary to aid solubility. Careful monitoring took place to ensure bile acids remained in solution for the duration of experiments.

Cell culture

The STC-1 clonal cell line was received as a kind gift from Dr. D. Hanahan (University of California, San Francisco, CA, USA). This enteroendocrine cell line originated from a double transgenic mouse tumour (Rindi et al., 1990). STC-1 cells were cultured in DMEM containing 4.5 g/l D-glucose, without sodium pyruvate (GlutaMAX, Gibco, Paisley, UK) and supplemented with 17.5% foetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/l streptomycin and incubated in an 5% CO_2 humidified atmosphere at 37°C. Cells underwent passage upon reaching 80–90% confluence and were used between passage numbers 15 and 50.

STC-1 cells transfected with a plasmid (pGIP/Neo) encoding neomycin phosphotransferase, driven by the GIP promoter (Ramshur et al., 2002), were obtained from Dr. B. Wice (Washington University of St. Louis, MO, USA) with permission from Dr. D. Hanahan (University of California, San Francisco, CA, USA). pGIP/Neo STC-1 cells were cultured in a similar manner to STC-1 cells, but media contained 10% FBS and was supplemented with Geneticin (G418, 400 μg/ml, Sigma).

Acute GLP-1 secretion in murine STC-1 cells

Approximately 2×10^6 cells were seeded into 12-well plates and incubated for 18 h at 37°C. Media were removed, the cells were washed with HEPES and then underwent 60 min preincubation with HEPES buffer (20 mM HEPES, 10 mM glucose, 140 nM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$). Buffer was aspirated off and cells were incubated for 3 h with 400 μl of vehicle or bile acid (100 μM) test solution. Following the test period, 350 μl of the incubation solution was removed to a separate tube, placed on ice and centrifuged (900 *g*, 5 min) to remove any cellular debris. The supernatant was collected and stored at -80°C prior to further analysis by radioimmunoassay. GLP-1 was measured in supernatant using an in-house fully optimised radioimmunoassay which used anti-rabbit IgG Sac-Cel (IDS, Boldon, UK) and a polyclonal antibody with zero cross-reactivity for glucagon or GIP.

Cellular LDH release

Cytotoxicity was determined using a LDH kit (Roche Diagnostics Ltd, West Sussex, UK). Briefly, STC-1 cells (5×10^4) were seeded into 96-well plates and cultured overnight at 37°C in a humidified atmosphere of 5% CO_2. Media were removed and fresh media (for controls) or bile acid supplemented media were added and cells were incubated for a period of 3 h. In accordance with the manufacturer's instructions, each experiment incorporated controls for background (LDH activity contained in the assay medium), spontaneous LDH release and maximum LDH release (Triton X-100). Following incubations, 5 μl of lysis solution was added to the media (n=8) and this was incubated for 15 min (37°C). Assay buffer (100 μl) was added for a further 30 min and this was followed by stop solution (50 μl). Finally, the plates now containing cells, media, lysis buffer and assay buffer were shaken and absorbance measured at 492 nm (reference wavelength=600 nm).

Acute GIP secretion in pGIP/Neo STC-1 cells

Before investigating bile acid responses pGIP/Neo STC-1 cells were characterised for their ability to produce and secrete GIP. Mean cellular GIP content was 2784±429 pmol/l/10^6 cells and GIP con-

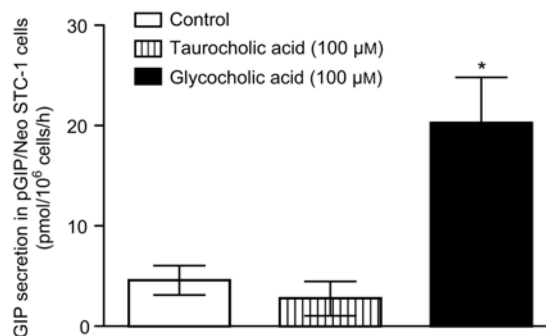

Figure 6 Effects of taurocholic, or glycocholic acid on acute GIP secretion in pGIP/Neo STC-1 cells pGIP/Neo STC-1 cells were incubated for 3 h with taurocholic or glycocholic acid (100 μM) before determination of GIP secretion.
Results are mean±SEM (n=12). *$p<0.05$ compared with vehicle control; ns, not significant.

centrations in cell culture media were 1659±170 pmol/l/10^6 cells. Secretory responsiveness to various fatty acids was also confirmed (data not shown). GIP secretion was determined by a similar method to GLP-1 secretion (above) but using 2×10^6 cells seeded into 12-well plates. GIP was measured using a commercial ELISA kit (Pheonix Pharmaceuticals, Belmont, CA, USA).

Animals

Mice lacking functional GLP-1 receptors (GLP-$1R^{-/-}$) (Scrocchi and Drucker, 1998; Ayala et al., 2010) were kindly provided by Dr. D.J. Drucker, University of Toronto, Ontario, Canada. Mice were of C57BL/6 genetic background and wild-type C57BL/6 mice (Harlan, Bicester, UK) were used for comparison. Mice aged 15–19 weeks were housed in an air-conditioned room at 22±2°C with a 12 h light (06:00–18:00)/12 h dark cycle (18:00–06:00). Drinking water and a standard rodent maintenance diet (Teklad global rodent diet, Harlan) were freely available. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of any of the compounds.

Oral glucose tolerance tests in normal and GLP-$1R^{-/-}$ mice

Normal or GLP-$1R^{-/-}$ mice were fasted for 16 h before oral gavage with glucose (18 mmol/kg), or glucose in combination with glycocholic acid (100 mg/kg) or taurocholic acid (100 mg/kg). In a second series of experiments assessing incretin receptor blockade, exendin(9–39) and (Pro^3)GIP are high-affinity antagonists of the GLP-1 receptor and the GIP receptor, respectively (Göke et al., 1993; Gault et al., 2003). These peptides have been individually employed to probe the physiological significance of these incretin receptors in various settings (Göke et al., 1993; Irwin et al., 2006; Parker et al., 2007; Green et al., 2008). On several previous occasions intraperitoneal doses ranging from 25 to 50 nmol/kg successfully counteract concomitant concentrations of GLP-1(7–36)amide or GIP(1–42) (Gault et al., 2003; Green et al., 2005; Irwin et al., 2010). Normal mice were given intraperitoneal saline (0.9%, w/v) or saline containing exendin(9–39) (50 nmol/kg) and GLP-$1R^{-/-}$ mice were given intraperitoneal saline (0.9% w/v), or saline containing (Pro^3)GIP (50 nmol/kg) immediately after oral gavage with glucose and taurocholic acid. Blood glucose was analysed immediately before administration and 15, 30, 60 and 105 min post-administration using a FreeStyle Blood Glucose Monitor (Abbott Laboratories Ireland Ltd., Dublin, Ireland). For studies measuring plasma GLP-1, mice were sacrificed in a carbon dioxide atmosphere 30 min post-administration and blood samples taken by cardiac puncture using a heparinised syringe. Blood was centrifuged for 30 s at 13 000 *g* (IEC Micromax RF) and plasma stored at -80°C prior to analysis.

Data analysis

Results were expressed as mean±SEM. Plasma glucose data were compared using the unpaired Student's *t*-test. AUC values were compared using repeated measures one-way analysis of variation followed by the Student-Newman-Keuls post-hoc test. Incremental areas under plasma glucose curves (ΔAUC0–105) were calculated using a computer-generated program employing the trapezoidal rule (Burington, 1973) with baseline subtraction. Groups of data were considered to be significantly different if $p<0.05$.

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