Bile Acids Induce Glucagon-Like Peptide 2 Secretion with Limited Effects on Intestinal Adaptation in Early Weaned Pigs

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Abstract

Early weaning is a stressful event characterized by a transient period of intestinal atrophy that may be mediated by reduced secretion of glucagon-like peptide (GLP) 2. We tested whether enterally fed bile acids or plant sterols could increase nutrient-dependent GLP-2 secretion and improve intestinal adaptation in weaning pigs. During the first 6 d after weaning, piglets were intragastrically infused once daily with either deionized water (control), chenodeoxycholic acid (CDC; 60 mg/kg body weight), or β-sitosterol (BSE; 100 mg/kg body weight). Infusing CDC increased plasma GLP-2 (P < 0.05) but did not affect plasma GLP-1 and feed intake. The intestinal expression of Glp2r (glucagon-like peptide 2 receptor), Asbt (sodium-dependent bile acid transporter), Fxr (farnesoid X receptor), and Tgr5 (guanosine protein–coupled bile acid receptor) genes were not affected by CDC treatment. The intragastric administration of CDC did not alter the weight and length of the intestine, yet increased the activation of caspase-3 in ileal villi (P < 0.02) and the expression of Il6 (interleukin 6; P < 0.002) in the jejunum. In contrast, infusing BSE did not affect any of the variables that were measured. Our results show that the enteral administration of the bile acid CDC potentiates the nutrient-induced secretion of endogenous GLP-2 in early-weaned pigs. Bile acid–enhanced release of GLP-2, however, did not result in improved intestinal growth, morphology, or inflammation during the postweaning degenerative phase.

Introduction

In most settings of pig production, piglets are abruptly separated from their mothers before intestinal development is completed and confronted with a number of physical and psychological stressors including drastic dietary changes and commingling with unfamiliar mates. As a result, newly weaned pigs become transiently anorectic and during the following 3 to 5 d experience severe deterioration of the intestinal mucosa structure and function, ultimately leading to reduced growth and increased susceptibility to diarrhea and enteric infections (1–4). Whereas the composition of the postweaning diet has little influence (5), the lack of enteral feeding (2) and activation of stress signaling pathways (6) appear to account for the pathology of early weaning (EW) (7).

Similar to EW, feeding neonatal pigs parenterally causes gut atrophy and impairs the enterohepatic circulation of bile acids (7,8). The former anomaly is partly explained by a reduction in the secretion of glucagon-like peptide (GLP) 2, a pleiotropic peptide cosecreted with GLP-1 from enteroendocrine L cells in response to luminal nutrients (9,10). In total parenteral nutrition (TPN)–fed pigs, administration of GLP-2 virtually restores growth of the intestinal mucosa by stimulating cell proliferation and suppressing apoptosis (9). In addition, exogenous GLP-2 reduces intestinal permeability in normal and psychologically stressed animals (11,12) and presents anti-inflammatory effects that are independent of its mitogenic action (13).
weaning-induced stress and anorexia are associated with a remarkable reduction in circulating GLP-2, it is possible that this regulatory peptide is also implicated in the loss of gut integrity caused by EW (14).

In the intestine, GLP secretion responds to nutritional, hormonal, and neural stimuli (9,10). With regard to hormonal signals, bile acids have recently emerged as potent regulators of GLP release by signaling through the guanosine protein–coupled bile acid receptor (TGR5), which is a bile acid–sensing receptor expressed on the luminal surface of enteroendocrine L cells (15). Upon absorption in the ileum by the sodium-dependent bile acid transporter (ASBT), bile acids bind and activate the nuclear receptor farnesoid X receptor (FXR), controlling thereby their own enterohepatic clearance through transcriptional and hormonal mechanisms (16). By antagonizing FXR action (17) or through upregulation of the liver X receptor–regulated genes (18), plant sterols may contribute to alter the enterohepatic circulation of bile acids in TPN-fed neonates (17,19) and, as a result, might indirectly modify the TGR5-mediated activation of the GLP pathway. Provided that plant sterols are able to antagonize FXR (17), they might also modulate GLP secretion via direct interaction with TGR5.

This study is based on the hypothesis that EW partly disrupts gut signaling cascades initiated by luminal nutrients and bile acids, and as a consequence, agonists of TGR5 may allow potentiating the nutrient-dependent secretion of GLP-2, thereby alleviating enteric disorders caused by EW. We thus evaluated the impact of the gastroduodenal supplementation with the bile acid chenodeoxycholic acid (CDC) or phytoesterol β-sitosterol (BSE) to EW pigs on circulating GLP-2 and intestinal growth, morphology, and inflammation.

Materials and Methods

Animals and housing. All experimental procedures were approved by the Laboratory Animal Care Advisory Committee of the Faculty of Veterinary Sciences of the Universitat Autònoma de Barcelona, Spain. A total of 36 pigs (18 of each sex; Large white × Landrace × Pietrain) were used in a study conducted at the Swine Experimental Unit of Lucta S.A. (Girona, Spain). Piglets were weaned at 22 kg of initial body weight (1; Sigma-Aldrich), and BSE (100 mg/kg) were administered once daily at 1900 as a single dose to limit animal handling during the lighting photoperiod and to avoid circular feeding. Upon absorption in the ileum by the sodium-dependent bile acid transporter (ASBT), bile acids bind and activate the nuclear receptor farnesoid X receptor (FXR), controlling thereby their own enterohepatic clearance through transcriptional and hormonal mechanisms. By antagonizing FXR action (17) or through upregulation of the liver X receptor–regulated genes (18), plant sterols may contribute to alter the enterohepatic circulation of bile acids in TPN-fed neonates (17,19) and, as a result, might indirectly modify the TGR5-mediated activation of the GLP pathway. Provided that plant sterols are able to antagonize FXR (17), they might also modulate GLP secretion via direct interaction with TGR5.

The dose of CDC used in this study was double the amount used by Jain et al. (20) to induce GLP secretion in neonatal piglets. In view of our objectives, this seemed reasonable because the intestinal sensitivity to GLP-2 is expected to decrease immediately after EW (14) and bile acids activate the GLP pathway in a dose-dependent manner (15). The dose of BSE was adjusted to be equivalent to the amount of phytosterols found to alter the enterohepatic circulation of bile acids in TPN-fed pigs (19).

Plasma collection and analysis. On day 5 after 12–14 h of food deprivation, blood samples were obtained from 6 randomly chosen pigs per treatment via jugular venipuncture at −15, 0, 30, 60, and 120 min relative to infusions. Samples were collected in tubes containing tri-potassium EDTA and aprotinin (BD Vacutainer), held in ice-cold water for 30 min, centrifuged at 2000 g for 10 min, stored frozen at −80°C, and analyzed as described previously for total GLP-1 (21) and GLP-2 (8).

Tissue collection. On day 6 after 3 h of food deprivation, the 6 pigs per treatment that did not previously undergo blood sampling were killed with an i.v. injection of sodium pentobarbital (200 mg·kg−1 body weight; Fatro Iberica). The abdomen was opened; the intestines were removed and dissected into sections designated as jejunum (from the pyloric sphincter to the first Peyer’s patch), ileum (from the first Peyer’s patch to the ileocecal valve), and large intestine (from the ileocecal valve to the rectum). Intestinal sections were measured, flushed with saline, and weighed. A 10-cm segment was removed from the midssection of the jejunum and ileum, divided into 5-cm halves, and opened longitudinally. Half of these samples were placed in RNA later (Applied Biosystems) and stored at −80°C until gene expression analysis, whereas the remaining samples were fixed in 10% buffered formalin for later histologic determination.

Morphometric analysis. Samples of jejunum and ileum were dehydrated and embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin. Villus height, crypt depth, percentage of crypt fission, number of intraepithelial lymphocytes in villi, and number of goblet cells in crypts were measured in 10 well-oriented villi and crypts by using a light microscope (Olympus) and a linear ocular micrometer (Olympus). All measurements were performed by the same person who was blinded to the treatments, as described previously (22,23).

Immunohistochemistry. Positive cells for cleaved caspase-3 were quantified to identify apoptotic cells in jejunum and ileum according to previous work (24). The marker Ki67 was quantified in jejunal and ileal crypts to detect proliferating enterocytes following the manufacturer’s instructions (Vector Labs). The marker cluster of differentiation (CD) 3 was used to measure T lymphocytes in ileal villi and CD79α was used to measure B lymphocytes in Peyer’s patches as previously described (25). All measurements were performed on formalin-fixed, paraffin-embedded tissue sections.

Real-time RT-qPCR analysis. Jejunal and ileal samples were thawed, and 50 mg of the mucosal scrapings were weighed, placed into vials containing 1 mL of Trizol (Sigma-Aldrich), and grinded with a mixer mill (MM-200; Restch). Total RNA was isolated by using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following the manufacturer’s instructions. A DNase treatment step using RNase-Free DNase Set (Qiagen) was added to prevent genomic DNA contamination. RNA yield and quality were determined spectrophotometrically by absorbance at the wavelengths of 260 and 280 nm. First-strand cDNA was synthesized by using the High-Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer’s instructions. qPCR was performed in an ABI Prism 7300 Sequence Detector System (Applied Biosystems) using SYBR Green (Molecular Probes) as a fluorescent DNA intercalating agent. Primers and optimal PCR conditions for the porcine GLP-2 receptor (Glp2r) (26), tumor necrosis factor α (Tnfa) (27), interleukin 6 (Il6) (27), II10 (28), leukemia inhibitory factor (Lif) (29), and the reference genes TATA-box binding protein (Tbp) (30) and β-actin (Actb) (31) were taken from the literature. Primers for porcine Fxr, Gcg (proglucagon), Asbt, and Tgr5 were designed (Supplemental Table 2). Specific product amplification was checked by agarose gel electrophoresis and through the melting curve analysis. All samples were conducted in triplicate in 96-well plates mixing the appropriate quantity of each primer with SYBR Green Master Mix (Applied Biosystems), ultrapurified water, and ~100 ng cDNA as template.
RESULTS

Body weight and intake. Pigs in all 3 groups—control, CDC, and BSE—grew to a similar extent and feed intake was not affected by treatments (Supplemental Table 3). During the study, no signs of adverse treatment effects were detected.

Secretion of GLP and expression of related genes. The intragastric administration of CDC elevated plasma GLP-2, which differed from the control group at the last sampling time ($P < 0.01$) (Fig. 1A). Relative to control, CDC infusion increased the mean plasma concentration of GLP-2 by $\approx 80\%$ ($P < 0.01$) (Fig. 1B). In contrast, mean plasma GLP-1 was not altered by CDC infusion (Fig. 1B). Compared with the control group, administration of BSE did not affect the plasma concentration of GLP-2 and GLP-1. The BSE group, however, had lower plasma GLP-2 and GLP-1 than did the CDC group (Fig. 1).

The relative expression of Gcg, which encodes GLP along with other hormones, and Gip2r, which encodes the guanosine protein–coupled receptor (GPCR) that mediates the pleiotropic actions of GLP-2, was not affected by treatments (Supplemental Fig. 1).

Expression of genes related to bile acid metabolism. The expression of Asbt, Tgr5, and Fxr was detected both in jejunum and ileum, but the relative abundance of their mRNA was similar among treatments in each section (Fig. 2A–C).

Intestinal growth, morphology, and cell proliferation. In comparison with controls, infusion of CDC tended to increase ($P < 0.08$) the weight and length of the ileum, whereas BSE did not affect the size of the intestines. Pigs in the BSE group, however, had a shorter small intestine ($P < 0.05$) than those in the CDC group (Table 1).

The administration of CDC and BSE did not modify the villus to crypt ratio, crypt fission, villus height, crypt depth, or the number of goblet cells in jejunum and ileum (Supplemental Table 3). Additionally, CDC did not promote mucosal cell proliferation but resulted in a greater ($P < 0.02$) degree of caspase-3 activation in ileal villi compared with control (Fig. 3A, B). Infusion of BSE increased the number of Ki67-positive cells in the jejunum ($P < 0.02$) and did not alter cell apoptosis when compared with control (Fig. 3A, B).

Intestinal inflammation. The histologic examination of samples revealed an elevated ($P < 0.04$) infiltration of lymphocytes in the ileal epithelium of CDC pigs compared with their control counterparts (Fig. 4A). For this reason, the inflammatory response was further investigated by using immunohistologic and RT-qPCR analyses. No differences in CD3 (T cell) and CD79a (B cell) labeling were found between control and CDC groups, whereas infiltration of CD79a+ lymphocytes was the largest ($P < 0.04$) in the BSE group (Fig. 4B, C). In comparison with the control and BSE, CDC infusion induced a greater expression of Il6 ($P < 0.002$) in the jejunum (Fig. 5B). No treatment effects were observed for Lif and Il10 (Supplemental Fig. 2).

Discussion

Intestinal pathologies caused by EW, such as impaired growth, permeability, and immune homeostasis of the mucosa, resemble those caused by TPN in neonatal pigs (7,8) and psychological stress in rodents (12). Furthermore, EW-induced anorexia is likely to interrupt the discharge of bile acids into the intestines as occurs in parenterally fed neonates (8). Under these circumstances, gut-signaling cascades controlling the release of GLP may be transiently disrupted, which could account for the reduction in circulating GLP-2 that is observed at weaning (14). Importantly, administration of exogenous GLP-2 to TPN-fed neonates or stressed animals restores gut integrity and ameliorates gut mucosal inflammation (9–12). Thus, we hypothesized...
that stimulating endogenous GLP-2 secretion over the response elicited by ingested nutrients may improve intestinal adaptation during EW.

Bile acids control GLP secretion via activation of the bile acid-sensing GPCR, TGR5 (15). Activating this GPCR with natural or synthetic agonists allowed augmenting the release of GLP in vitro and in vivo (15,33). In addition, phytosterols are known to interact with the bile acid receptor FXR (17). This property makes them possible candidates for modulating the activation of GLP pathway via altered enterohepatic circulation of bile acids and/or direct interaction with TGR5. Thus, we predicted that targeting gut signaling pathways controlling the secretion of GLP with ligands of the receptor TGR5 may allow potentiating the nutrient-dependent secretion of GLP-2 during EW.

To test the above hypotheses, EW piglets were infused intragastrically with CDC and BSE during the postweaning degenerative phase. As observed in TPN-fed piglets (20), infusion of CDC significantly increased mean plasma GLP-2 in our study. This response is in line with the ability of CDC to bind and activate the bile acid sensor TGR5 (34). We also show here novel evidence that Tgr5 is expressed in the proximal and distal small intestine of pigs and that CDC induction of GLP-2 did not involve changes in the expression of Tgr5 or Fxr. More important, our results support the hypothesis that targeting intestinal TGR5 with agonists such as bile acids can stimulate

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**TABLE 1** Intestinal weight and length of piglets infused intragastrically with control, CDC, or BSE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>CDC</th>
<th>BSE</th>
<th>SEM</th>
<th>P &gt; F</th>
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<tr>
<td>Organ weight, g/kg BW</td>
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<tr>
<td>Jejunum</td>
<td>28.7</td>
<td>34.8</td>
<td>28.4</td>
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<td>Ileum</td>
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<td>8.6</td>
<td>6.1</td>
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<td>0.08</td>
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<tr>
<td>Small intestine</td>
<td>35.2</td>
<td>43.4</td>
<td>35.2</td>
<td>3.66</td>
<td>0.19</td>
</tr>
<tr>
<td>Large intestine</td>
<td>13.5</td>
<td>15.2</td>
<td>13.1</td>
<td>1.19</td>
<td>0.46</td>
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<tr>
<td>Whole intestine</td>
<td>48.8</td>
<td>58.6</td>
<td>47.6</td>
<td>4.62</td>
<td>0.22</td>
</tr>
<tr>
<td>Organ length, cm/kg BW</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Jejunum</td>
<td>113</td>
<td>123</td>
<td>104</td>
<td>5.48</td>
<td>0.08</td>
</tr>
<tr>
<td>Ileum</td>
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<td>1.40</td>
<td>0.08</td>
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<tr>
<td>Small intestine</td>
<td>132</td>
<td>145</td>
<td>121</td>
<td>5.87</td>
<td>0.04</td>
</tr>
</tbody>
</table>

1 Values are least-squares means unless otherwise indicated, n = 6 per treatment. Labeled means in a row without a common letter differ, P < 0.05. BSE, β-sitosterol; BW, body weight; CDC, chenodeoxycholic acid.
2 Deionized water.
3 F-test statistic.

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**FIGURE 2** Relative concentrations of Asbt (A), Tgr5 (B), and Fxr (C) mRNA in the small intestine of piglets infused intragastrically with CDC or BSE. Values are least-squares means ± SEMs expressed as fold-changes relative to CON, n = 6. Asbt, sodium-dependent bile acid transporter; BSE, β-sitosterol; CDC, chenodeoxycholic acid; CON, control (deionized water); Fxr, farnesoid X receptor; Tgr5, guanosine protein-coupled bile acid receptor.

**FIGURE 3** Markers of cell proliferation (A) and apoptosis (B) in the small intestinal mucosa of piglets infused intragastrically with CON, CDC, or BSE. Values are least-squares means ± SEMs, n = 6. Labeled means without a common letter differ, P < 0.02. BSE, β-sitosterol; CDC, chenodeoxycholic acid; CON, control (deionized water); Ki67, marker of cell proliferation.
endogenous GLP-2 secretion in EW pigs fed standard cereal-based diets.

Contrary to our expectations, BSE was ineffective in increasing the concentration of circulating GLP-2 and improving gut adaptation to EW. In addition, the infusion of BSE did not affect the expression of \( \text{Fxr} \) and its target gene \( \text{Asbt} \). Even though stigmasterol, a soy-derived phytoesterol, antagonizes hepatic FXR [17], our results suggest that BSE did not interact with intestinal FXR and TGR5.

Enteral administration of CDC to TPN-fed newborn pigs promoted intestinal growth and villus elongation presumably by inducing the release of endogenous GLP-2 [20]. Despite the remarkable increases in plasma GLP-2 recorded in our study, CDC tended to enhance only ileal growth. Furthermore, we did not detect increased mucosal mitogenesis or reduced apoptosis, which are the distinctive cellular actions of GLP-2 [9,10]. On the contrary, CDC showed proapoptotic (i.e., increased active caspase-3) and proinflammatory (i.e., increased \( \text{Il6} \) expression) effects in the small intestine. These findings, along with the previous observation that the administration of exogenous GLP-2 at pharmacologic doses to piglets did not prevent weaning-induced gut atrophy [35], support the suggestion that the porcine intestine is more responsive to GLP-2 during the neonatal phase than after weaning [14]. It cannot be ruled out, however, that other negative bile acid–associated effects might have antagonized the trophic actions and anti-inflammatory effects of endogenous GLP-2 on the intestine.

The pleiotropic actions of GLP-2 are mediated by the receptor GLP-2R, which is a GPCR predominantly distributed in the intestine [9,10]. In pigs, the resumption of feed ingestion after weaning-induced anorexia increases circulating GLP-2 but downregulates the intestinal expression of \( \text{Glp2r} \) [14]. In our study, however, CDC induction of GLP-2 did not affect the abundance of mRNA transcripts of \( \text{Glp2r} \) in the jejunum and ileum, suggesting that such a mechanism could not have accounted for its negligible impact on gut mass and mucosal architecture. Alternatively, evidence is mounting that GLP-2 exerts its actions indirectly via an increasing number of mediators [36]. Thus, one cannot preclude that the apparent decrease in GLP-2 responsiveness by the intestines after weaning may involve signaling pathways downstream of the GLP-2/GLP-2R axis.

On the basis of the expectation that the intestinal sensitivity to GLP-2 would decrease immediately after EW [14], we decided to use a 2 times higher dose of CDC than the one administered to neonate piglets by Jain et al. [20]. This seemed to be reasonable...
because the activation of the GLP pathway by bile acids (15) and the mitogenic action of GLP-2 (37) are dose-dependent processes. Nevertheless, we found that CDC infusion caused mucosal inflammation and emerging evidence indicates that diminished microbial transformation of primary into secondary bile acids in the large bowel is associated with intestinal inflammation (38). Consequently, we speculate that the bile acid form, dosage, and administration procedure (single dose) used in our studies resulted in a transient excess of CDC in the distal intestine that may have antagonized the beneficial effects of increased TGR5-mediated GLP-2 secretion.

Although the contribution of endogenous GLP-2 to generate satiety remains unclear (9,10), the anorectic properties of the cosecreted GLP-1 are well established (39). Therefore, a potential caveat of stimulating GLP-2 secretion during the postweaning degenerative phase is the risk of extending the period of suppressed feed intake that follows EW. We observed that the infusion of CDC increased circulating GLP-1 compared with BSE. Nonetheless, the latency to eat after weaning (data not shown) and the amount of feed consumed by piglets during the following 4 d were similar for control, CDC, and BSE groups. Furthermore, the 3 groups of pigs achieved amounts of feed consumption that resemble those observed under current settings of pig production (40). On the basis of these findings, it is tempting to suggest that mechanisms similar to the ones discussed for GLP-2 might have been implicated in reducing GLP-1 sensitivity immediately after weaning.

We conclude that the enteral administration of the bile acid CDC potentiates the nutrient-induced secretion of endogenous GLP-2 in EW pigs. Bile acid–enhanced release of GLP-2, however, did not result in improved intestinal growth, morphology, or inflammation during the postweaning degenerative phase.

Acknowledgments
I.R.I. and D.G.B. designed the research; I.R.I., G.T., D.M., N.d.D.C., J.J.H., M.N., and A.M. conducted the research; I.R.I., G.T., D.M., N.d.D.C., and A.M. analyzed the data; I.R.I., D.M., J.J.H., M.N., and D.G.B. wrote the manuscript; and I.R.I. had primary responsibility for the final content. All authors read and approved the final manuscript.

Literature Cited


