Intestine-selective reduction of Gcg expression reveals the importance of the distal gut for GLP-1 secretion

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ABSTRACT

Objective: Glucagon-like peptide-1 is a nutrient-sensitive hormone secreted from enteroendocrine L cells within the small and large bowel. Although GLP-1 levels rise rapidly in response to food ingestion, the greatest density of L cells is localized to the distal small bowel and colon. Here, we assessed the importance of the distal gut in the acute L cell response to diverse secretagogues.

Methods: Circulating levels of glucose and plasma GLP-1 were measured in response to the administration of L cell secretagogues in wild-type mice and in mice with (1) genetic reduction of Gcg expression throughout the small bowel and large bowel (GcgDistalGut-/-) and (2) selective reduction of Gcg expression in the distal gut (GcgDistalGut-/-).

Results: The acute GLP-1 response to olive oil or arginine administration was markedly diminished in GcgDistalGut-/- but preserved in GcgDistalGut-/- mice. In contrast, the increase in plasma GLP-1 levels following the administration of the GPR119 agonist AR231453, or the melanocortin-4 receptor (MC4R) agonist LY2112688, was markedly diminished in the GcgDistalGut-/- mice. The GLP-1 response to LPS was also markedly attenuated in the GcgDistalGut-/- mice and remained submaximal in the GcgDistalGut-/- mice. Doses of metformin sufficient to lower glucose and increase GLP-1 levels in the Gcg+/+ mice retained their glucoregulatory activity, yet they failed to increase GLP-1 levels in the GcgDistalGut-/- mice. Surprisingly, the actions of metformin to increase plasma GLP-1 levels were substantially attenuated in the GcgDistalGut-/- mice.

Conclusion: These findings further establish the importance of the proximal gut for the acute response to nutrient-related GLP-1 secretagogues. In contrast, we identify essential contributions of the distal gut to (i) the rapid induction of circulating GLP-1 levels in response to pharmacological selective agonism of G-protein-coupled receptors, (ii) the increased GLP-1 levels following the activation of Toll-like receptors with LPS, and (iii) the acute GLP-1 response to metformin. Collectively, these results reveal that distal gut Gcg+ endocrine cells are rapid responders to structurally and functionally diverse GLP-1 secretagogues.

Keywords Peptides; Intestine; Pancreas; Diabetes; Obesity; Metformin; Inflammation

1. INTRODUCTION

The gastrointestinal enteroendocrine system contains dozens of phenotypically distinct plurihormonal cell types linking signals from ingested nutrients and bacterial metabolites to the control of peptide hormone secretion [1,2]. Among the best characterized enteroendocrine cells, the L cell, notable for the synthesis and secretion of multiple proglucagon-derived peptides (PGDPs), has received considerable scrutiny. Indeed, posttranslational processing of proglucagon in the small intestine and large intestine yields glicentin, oxyntomodulin, GLP-1 and GLP-2 [3,4]. PGDPs with roles in the regulation of gut motility, mucosal integrity, nutrient absorption, the control of appetite, and nutrient assimilation [5]. Moreover, the clinical development of GLP-1R agonists for the treatment of diabetes and obesity and a GLP-2R agonist for the therapy of intestinal failure [6] has heightened interest in understanding the translational biology of gut PGDP secretion and action.

The analysis of the location and distribution of L cells suggests an increasing gradient of the L cell number and PGDP content from the proximal gut to the distal gut, with the highest levels of Gcg mRNA transcripts and PGDPs being detected within the terminal ileum and colon [7-9]. Paradoxically, however, plasma levels of gut PGDPs, exemplified by GLP-1, increase within minutes of food ingestion, timing inconsistent with the notion that ingested complex macronutrients would be enzymatically digested and transported to the distal gut. Accordingly, several competing theories have evolved to reconcile
these observations. First, considerable evidence, largely from preclinical studies, supports the existence of a proximal-distal gut axis, whereby neural or hormonal signals are rapidly conveyed to distal gut L cells enabling GLP-1 secretion [10]. A second hypothesis invokes the functional importance of proximal gut L cells in the jejunum as sufficient to generate a rapid initial rise in GLP-1 secretion accounting for increased circulating GLP-1 levels within minutes of food intake [9,11,12].

More recently, the putative importance of pancreatic islet GLP-1 has received renewed attention. Although the levels of processed bioactive GLP-1 are very low in the normal mouse and human pancreas [13], islets examined ex vivo secrete GLP-1 [14]. Moreover, the development of diabetes and/or pancreatic injury has been associated with increased expression of prohormone convertase-1 (Pcsk1) in islet α-cells, accompanied by the enhanced biosynthesis and liberation of bioactive islet GLP-1 [15]. Strikingly, mice with selective reactivation of Gcg expression in the pancreas reveal an important glucoregulatory role for islet glucagon and/or GLP-1 production [16], rekindling interest in the physiological and pathological circumstances under which the pancreatic islets may represent an important source of glucoregulatory PGDPs, including GLP-1.

To better understand the relative role of the proximal gut and the distal gut in the generation of circulating GLP-1, we recently generated lines of mice with substantial elimination of Gcg expression in both the small intestine and large intestine (GcgDistal−/−) or more selective loss of distal gut Gcg expression in the terminal ileum and colon (GcgDistalIleum−/−) [13]. The analysis of these mice reinforced the importance of the gut as the predominant source of circulating GLP-1. Unexpectedly, circulating levels of GLP-1 were also lower in the fasting state, and glucose tolerance was impaired in GcgDistalIleum−/−mice [13], prompting questions about the relative contributions of the proximal and distal gut to the control of GLP-1 levels in the interprandial state and following nutrient ingestion. Here, we examined the contribution of distal gut Gcg expression to the acute response to ingested nutrients such as the amino acid arginine and olive oil, as well as pharmacological administration of an oral GPR119 agonist and parenteral administration of a melanocortin 4 receptor (MC4R) agonist, lipopolysaccharide (LPS), and metformin. Our findings reveal the unexpected importance of the distal gut Gcg system for the rapid initial response to functionally diverse L cell secretagogues.

2. MATERIALS AND METHODS

2.1. Animals

All studies were conducted in accordance with protocols approved by the Sinai Health System and The Centre for Phenogenomics (TCP, Toronto, ON, Canada). In vivo studies were performed predominantly in adult male mice beginning at 12 weeks old. As we did not observe sex-specific differences in secretagogue responses, in some cases, littermate-matched female mice were also used as appropriately noted in Figure Legends. The mice were housed in groups of up to five in microisolator cages in a pathogen-free facility on a 12/12 light–dark cycle. All animals had ad libitum access to irradiated rodent chow (18% kcal from fat, Harlan Teklad, Mississauga, ON, Canada) and sterile water unless otherwise noted. GcgDistal−/−, GcgDistalIleum−/−, and their littermate control +/+ mice were generated and genotyped as described previously [13,16]. Following the conclusion of experiments, Gcg knockdown was assessed in segments of the gut and pancreas to verify the expression and to remove animals with unintended germline deletion, as described previously [13].

2.2. Acute in vivo studies

To assess rapid plasma GLP-1 responses to specified secretory agents (Table 1), mice were subjected to acute experiments to detect peak plasma GLP-1 levels independent of normal food intake. Mice were fasted overnight (~16 h) in wire-bottom cages to minimize the ingestion of feces and bedding, with normal access to water. After the fasting period, mice were given a single bolus of the secretagogue by either oral gavage or intraperitoneal injection. Blood glucose was measured using a glucometer (Aviva glucometer, Accu-Chek, Roche, Toronto, ON, Canada), and blood was collected in lithium-heparin-coated capillary Microvette tubes (Sarstedt, Inc.) at the specified times, including at time 0, immediately before secretagogue treatment. The blood was quickly mixed with 10% EDTA (vol/vol) (5,000 IU/mL aprotinin) (Sigma A6279, CAS #9087-70-1), 1.2 mg/mL EDTA, and 0.1 mMol/L diprotin A (Sigma D3822, CAS #90614-45-5). Plasma was then isolated by centrifugation and then stored at −80 °C until subsequent hormone analysis.

2.3. Glucose tolerance tests

Prior to testing, mice were fasted overnight (~16 h). Using a triple-crossover study design, all mice were randomized to receive an oral gavage of water (vehicle), 50 mg/kg metformin, or 150 mg/kg metformin. Treatments were switched for subsequent tests, occurring 2 weeks apart for sufficient recovery/washout, so that all animals received all treatments. Sixty minutes after treatment, a glucose bolus was provided. A dose of 2 g/kg glucose in water (Sigma, catalog# G8270) was used for oral glucose tolerance tests (oGTTs), and 1.5 g/kg glucose in PBS was used for intraperitoneal glucose tolerance tests (iPGTs). Blood glucose was monitored at specified time points, and ~60 μL of the whole blood was collected via tail vein at time 0 (relative to glucose administration), 15 min, and 60 min and mixed with 10% TED. Plasma was separated and stored at −80 °C for subsequent hormone analysis.

2.4. Hormone assays

Plasma samples frozen at −80 °C were thawed on ice on the day of hormone analyses. Insulin was measured using the Mouse Ultrase nsitive Insulin ELISA (Alpco, 80-INSMSU-E01, 5 μL volume). Total GLP-1 was measured using V-PLEX GLP-1 Total Kit (Mesoscale Discovery, K1503PD, 25 μL volume). Unknowns were extrapolated from standard curves run in duplicate, according to provided protocols.

2.5. Data analysis

All graphs were produced and data analyzed using GraphPad Prism 7.0e. All graphical values are presented as mean ± SD. Statistical significance was calculated using either a two-tailed t-test or ANOVA with paired Tukey’s multiple comparison test, where appropriate. A P value < 0.05 was considered statistically significant.

3. RESULTS

To elucidate the importance of the distal gut in acute GLP-1 secretion, we studied GcgDistal−/− and GcgDistalIleum−/− mice and their respective wild-type littermate controls. GcgDistal−/− mice were generated by crossing GcgFlloxFllox mice with Vili-Cre mice and exhibit markedly reduced Gcg expression in both small bowel and large bowel [13]. GcgDistalIleum−/− mice were generated by crossing GcgFlloxFllox mice with Cdx2-Cre mice and display substantial attenuation of Gcg expression in the distal ileum and colon [13]. For all experiments, wild-type, GcgFlloxFllox, Cdx2-Cre, or Vili-Cre littermates were pooled and studied as controls. To assess the consequences of reduced Gcg expression on the secretory
capacity of gut L cells, we focused on GLP-1 due to its metabolic importance and the simultaneous availability of sensitive validated assays for the detection of circulating GLP-1 in mice [17].

3.1. Distal Gut Gcg expression is dispensable for nutrient-stimulated increments in plasma GLP-1

Prior studies determined that the secretion of GLP-1 following oral glucose challenge originates predominantly from the proximal gut [13]. We then assessed the GLP-1 levels in response to olive oil or arginine, known as stimulators of PGDP secretion [17]. Olive oil administered by oral gavage produced a modest rise in blood glucose excursion, consistent with a modest stress response, which was similar across all genotypes (Figure 1A,B, E, and F). In contrast to robust plasma GLP-1 levels in response to arginine, which was similar across all genotypes (Figure 1K and L). In contrast, plasma GLP-1 levels were different in GcgDistalGut+/+ versus GcgDistalGut−/− mice (Figure 1G,H), revealing that distal gut Gcg expression is not required for acute increments in plasma GLP-1 levels after lipid ingestion. We next challenged mice with oral arginine, an amino acid known to potentiate stimulate GLP-1 secretion [18]. Blood glucose levels rose modestly following arginine gavage in all genotypes (Figure 1I, J, M, and N). Notably, GcgDistalGut−/− mice exhibited a markedly reduced plasma GLP-1 excursions in response to arginine (Figure 1K and L). In contrast, maximal GLP-1 levels were not different among GcgDistalGut+/+ and GcgDistalGut−/− mice (Figure 1G,H), revealing that the absence of distal gut Gcg expression is not required for acute increments in plasma GLP-1 levels after lipid ingestion. Taken together with prior studies of glucose-stimulated GLP-1 secretion [13], our results suggest that LPS-mediated GLP-1 secretion originates from the gut, with a component of the response arising from the distal gut.

3.2. Distal Gut Gcg expression is required for GPR119 and MC4R agonist-stimulated increases in plasma GLP-1 levels

We next examined the intestinal sites important for the transduction of GLP-1 secretory signals pursuant to the activation of two L-cell-associated G-protein-coupled receptors (GPCRs). GPR119 is activated by multiple derivatives of dietary fatty acids and regulates metabolism in part via the stimulation of incretin secretion [19−21]. Oral gavage of the GPR119 agonist, AR231453, had no meaningful effect on the glycemic excursion in GcgDistalGut+/+ versus GcgDistalGut−/− mice (Figure 2A,B). Surprisingly, the rise in plasma GLP-1 levels after oral AR231453 was markedly attenuated in GcgDistalGut−/− mice, implicating the importance of the distal gut for maximal L cell responses to acute GPR119 agonism (Figure 2C,D). The MC4R is a GPCR expressed primarily in the brain, yet MC4R has also been detected outside the central nervous system, including within mouse and human L cells [22]. Intraportal injection of the MC4R-selective agonist LY2112688 had little impact on blood glucose levels in GcgDistalGut+/+ and GcgDistalGut−/− mice (Figure 2E,F). Plasma GLP-1 levels were increased following LY2112688 in GcgDistalGut−/− mice, yet there was no GLP-1 response in GcgDistalGut+/+ mice (Figure 2G,H). Hence, maximal GLP-1 excursions to either GPR119 or MC4R agonism require Gcg expression within the distal gut.

3.3. LPS requires distal Gut Gcg expression for maximal increases in plasma GLP-1

There is considerable evidence that links the administration of bacteria-derived LPS to the augmentation of L cell GLP-1 secretion in mice and humans [23,24]. Intraperitoneal injection of LPS produced a modest reduction in blood glucose levels 3 h after treatment to a similar extent in all genotypes tested (Figure 3A,B, E, and F). The increase in plasma GLP-1 after LPS was clearly dependent on gut Gcg expression as it was virtually extinguished in GcgDistalGut−/− mice (Figure 3C,D). Intriguingly, the increase in plasma GLP-1 in LPS-treated mice remained blunted in GcgDistalGut−/− mice (Figure 3G,H). These results suggest that LPS-mediated GLP-1 secretion originates from the gut, with a component of the response arising from the distal gut.

3.4. Distal Gut Gcg expression is essential for acute metformin-induced rises in plasma GLP-1

Among the many actions of metformin that may contribute to its glucoregulatory properties is the enhancement of gut GLP-1 secretion [25−27]. Oral metformin administration at a dose of 150 mg/kg produced a small reduction in blood glucose that was similar across genotypes (Figure 4A,B, E, and F). Notably, metformin failed to increase plasma GLP-1 levels in GcgDistalGut−/− mice (Figure 4C,D). Unexpectedly, metformin also did not increase plasma GLP-1 levels in GcgDistalGut−/− mice (Figure 4G,H). Hence, distal gut Gcg expression is required for the acute metformin-induced increment in plasma GLP-1.

3.5. Gut Gcg expression and increases in plasma GLP-1 are not required for metformin-mediated glucoregulation

To determine if a metformin-induced increment in plasma GLP-1 was required for glucoregulation, we treated GcgDistalGut+/+ and GcgDistalGut−/− mice with oral metformin at doses of 50 and 150 mg/kg 60 min prior to an oral (oGTT) and intraperitoneal glucose tolerance tests (ipGTT). In the context of an oGTT, a dose-dependent reduction in blood glucose was observed in GcgDistalGut+/+ mice (Figure 5A,B). The various metformin doses did not impact insulin levels (Figure 5C); however, plasma GLP-1 levels were increased after metformin administration (Figure 5D). Notably, plasma glucose and insulin responses to metformin were similar in GcgDistalGut+/+ and GcgDistalGut−/− mice (Figure 5E−G), despite the lack of increase in plasma GLP-1 levels in GcgDistalGut−/− mice (Figure 5H). Consistent with the oGTT results, metformin lowered glucose during an

Table 1 — GLP-1 secretagogues.

| Treatment       | Description          | Route of Admin | Vehicle | Dose            | Manufacturer (CAS#) |
|-----------------|----------------------|----------------|---------|-----------------|---------------------|
| Olive oil       | Macronutrient        | Oral           | None    | 200 μL          | Sigma 01514 (8000-25-0) |
| AR231453        | Amino acid           | Oral           | Water   | 2 g/kg          | Sigma AS506 (74-79-3) |
| GPR119 agonist  | Oral                 | 80% Polyethylene glycol-400, 10% Tween-80, 10% ethanol | 10 mg/kg | PEG 400, Sigma P3265 (25322-83-3) Tween-80 |
| LY2112688      | Melanocortin-4 receptor (MC4R) peptide agonist | Intraperitoneal | PBS | 3 mg/kg | Sigma P4780 (8005-65-6) Bachem (819048-44-7) |
| Lipo polysaccharide | O55:B5, O111:B4 | Inflammatory stimulus | Intraperitoneal | PBS | 1 mg/kg | Sigma L2860 and L3024 |
| Metformin      | Common diabetes drug | Oral           | Water   | 50 and 150 mg/kg | MP Biomedicals, Solon OH, (1115-70-4) |
intraperitoneal glucose challenge independent of the changes in plasma GLP-1 (Figures 5I–5P). Taken together, these results suggest that the acute glucoregulatory actions of metformin do not require an increase in plasma levels of GLP-1.

4. DISCUSSION

The findings that Gcg mRNA transcripts and corresponding levels of PGDPs, including GLP-1, are distributed throughout the small bowel...
Figure 2: Distal gut Gcg expression is required for acute GPR119- and MC4R-stimulated increases in plasma GLP-1. Gcg<sup>D<sub>istalGut</sup>+/+</sub> and Gcg<sup>D<sub>istalGut</sub></sup>−/− mice were treated with either an oral gavage of the GPR119 agonist, AR231453 (10 mg/kg BW), vehicle alone, or an intraperitoneal injection of the MC4R agonist, LY2112688 (3 mg/kg BW), at time zero. Blood glucose and total GLP-1 (tGLP-1) were measured at the indicated time points. (A, B) Time course of blood glucose levels during AR231453 stimulus and corresponding AUC analysis. (C, D) Time course of total tGLP-1 levels during AR231453 stimulus and corresponding AUC analysis. (E, F) Time course of blood glucose levels during LY2112688 stimulus and corresponding AUC analysis. (G, H) Time course of total tGLP-1 levels during LY2112688 stimulus and corresponding AUC analysis. For AR231453 studies: Gcg<sup>D<sub>istalGut</sub></sup>+/+, AR231453, n = 14 (males + females); Gcg<sup>D<sub>istalGut</sub></sup>−/− Vehicle, n = 5 (males + females); Gcg<sup>D<sub>istalGut</sub></sup>−/− AR231453, n = 5 (males + females). For LY2112688 studies: Gcg<sup>D<sub>istalGut</sub></sup>−/−, n = 19 (males + females); Gcg<sup>D<sub>istalGut</sub></sup>−/−, n = 5 (males + females). Statistical significance was determined using one-way ANOVA (panels B, D) or two-tailed t-test (panels F, H). **P < 0.01; ***P < 0.001.

and large bowel have engendered considerable debate toward understanding the relative importance of the proximal and the distal guts in the control of rapid meal-stimulated increases in circulating levels of GLP-1. Considerable evidence suggests that nutrient-stimulated hormonal mediators such as glucose-dependent insulinotropic polypeptide (GIP) liberated from the proximal gut or signals conveyed via neural transmission involving acetylcholine and gastrin-releasing peptide contribute to the amplification of L cell GLP-1 secretion from the distal gut in preclinical studies [10]. Furthermore, luminal perfusion of the proximal and the distal small bowel revealed a considerably greater increment in circulating GLP-1 following the stimulation of distal versus proximal gut L cells [28]. On the other hand, the levels of Gcg mRNA transcripts and GLP-1 content in the human proximal small bowel were not substantially different from those found more distally, highlighting potential contributions of the proximal small bowel to GLP-1 biosynthesis and secretion [9]. Moreover, the capacity of the isolated proximal rat gut to secrete GLP-1 in response to GRP or luminal peptone was not meaningfully different relative to the amounts of GLP-1 secreted by distal gut segments in acute short-term studies [12]. Hence, these contrasting findings illustrate the challenges in the interpretation of the relative importance of the proximal and the distal gut for the acute GLP-1 response to various secretagogues.

To better understand the contributions of the distal gut to the acute increase in circulating GLP-1 levels evident following the administration of nutrients and pharmacological agents, we employed mouse lines engineered to exhibit a substantial reduction of the Gcg expression throughout the small bowel and large bowel or predominately in the terminal ileum and colon [13]. We previously determined that GLP-1 levels rose briskly following oral administration of glucose to Gcg<sup>D<sub>istalGut</sub></sup>−/− mice, indirectly affirming the functional competence of proximal gut L cells for maximal glucose-regulated GLP-1 secretion. The current studies using the administration of olive oil or arginine further substantiate the redundancy of distal gut L cells for nutrient-stimulated GLP-1 secretion, as plasma levels of GLP-1 were not substantially different after these nutrient challenges in Gcg<sup>D<sub>istalGut</sub></sup>−/− versus Gcg<sup>D<sub>istalGut</sub></sup>−/−. In marked contrast, plasma GLP-1 responses to the GPR119 agonist AR231453, or the MC4R agonist LY2112688, were substantially attenuated in Gcg<sup>D<sub>istalGut</sub></sup>−/− mice. A functional GPR119 recognized by several lipid-related ligands, including cannabinoids, monoacylglycerols such as 2-oleoyl glycerol (2-OG), and oleoylethanolamide, is expressed within murine enteroendocrine L cells in the small bowel and large bowel [29,30]. However, the relative contributions of regional L cell populations to the rise in circulating GLP-1 levels following acute GPR119 activation have not previously been determined. To answer this question, we used the validated pharmacological GPR119 agonist AR231435, an agent we and others have previously shown to be highly selective for the GPR119 receptor [19–21]. Remarkably, although AR231453 was previously shown to stimulate the secretion of GIP, a hormone predominantly secreted from the proximal intestine [19,20], plasma levels of GLP-1 did not rise in response to oral AR231453 in Gcg<sup>D<sub>istalGut</sub></sup>−/− mice. Intriguingly, the
levels of GLP-1 in circulating levels of peptide YY and GLP-1 [22]. Consistent with these functionally linked to the secretion of L cell peptides and increased arising from genetic mutations in the MC4R pathway [34]. MC4R has use of GLP-1R agonists in the treatment of human subjects with obesity independent pathways [32,33], providing a rationale for the successful and rats demonstrated that GLP-1 reduces food intake through MC4R- including control of food intake and energy expenditure. Studies in mice MC4R is an extensively studied receptor mediating the regulation of food dependent GLP-1 secretion.

importance of the distal gut L cell as an important target for GPR119- including control of food intake and energy expenditure. Studies in mice including control of food intake and energy expenditure. Studies in mice

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stimulation of GLP-1 secretion by enteral olive oil, while appearing to be mediated through the activation of proximal gut L cells in the current experiments, might theoretically have required contributions to the GLP-1 secretion derived from lipid metabolites acting through GPR119 in the distal gut. Collectively, our findings are consistent with established actions of GPR119 agonists acting predominantly through mouse and human colonic L cells [30,31] and further highlight the importance of the distal gut L cell as an important target for GPR119-dependent GLP-1 secretion.

MC4R is an extensively studied receptor mediating the regulation of food intake and body weight through central and peripheral mechanisms including control of food intake and energy expenditure. Studies in mice and rats demonstrated that GLP-1 reduces food intake through MC4R-independent pathways [32,33], providing a rationale for the successful use of GLP-1R agonists in the treatment of human subjects with obesity arising from genetic mutations in the MC4R pathway [34]. MC4R has also been localized to enteroendocrine L cells, most notably in the colon, functionally linked to the secretion of L cell peptides and increased circulating levels of peptide YY and GLP-1 [22]. Consistent with these findings, the MC4R agonist LY2126688 rapidly increased the circulating levels of GLP-1 in Ggcfl/fl mice but not in Ggcfl/fl mice. Hence, although MC4R is expressed within cell populations of the stomach, small bowel, and large bowel [22], the distal gut is required for the acute GLP-1 response to the MC4R agonism. Beyond roles in transduction of nutrient-related signals, substantial evidence supports a simultaneous role for L cells as pathogen sensors [35], linking inflammatory signals, including bacterial metabolites and cell wall products exemplified by LPS, to GLP-1 secretion in animals and humans [23,24,36]. Notably, transient vascular ischemia and mesenteric injury in the proximal small bowel of mice and human subjects produce a rapid rise in plasma GLP-1 levels [23]. Furthermore, LPS increased GLP-1 secretion from small bowel-derived STC-1 L cells [23], raising the possibility that both the small and large bowel L cells are capable of sensing tissue injury and inflammation linked to the enhanced GLP-1 secretion. Our findings reveal that the LPS-induced increment in circulating GLP-1 levels was attenuated, but not abolished, in Ggcfl/fl mice. Hence, it seems likely that LPS engages L cells in both the proximal and distal guts to enhance GLP-1 secretion.

The pleiotropic actions of metformin have also been linked to the acute GLP-1 secretion in preclinical studies [37] and in humans with type 2 diabetes [25]. Consistent with the importance of the gastrointestinal tract as a site of metformin action, gut-targeted metformin produces a rapid rise in plasma GLP-1 levels detectable within 60 min of metformin administration [38]. Moreover, intraduodenal metformin administration rapidly lowered glycemia and hepatic glucose production in rats through GLP-1R-dependent mechanisms [39], further highlighting the potential importance of the small bowel as a target for metformin-GLP-1 interactions. Nevertheless, our current findings reveal that low doses of enteral metformin are still capable of lowering glucose independent of any detectable changes in plasma GLP-1 levels in Ggcfl/fl mice. These observations are consistent with previous results demonstrating the preservation of the glucoregulatory actions of a range of metformin concentrations in Glp1r−− mice [27]. Although
Figure 4: Distal gut Gcg expression is essential for acute metformin-induced rises in plasma GLP-1. Blood glucose and total GLP-1 (tGLP-1) levels in Gcg<sup>fl/fl</sup>, Gcg<sup>gut−/−</sup>, and littermate control mice following oral gavage of metformin (150 mg/kg BW). (A, B) Time course of blood glucose levels during metformin stimulus and corresponding AUC analysis in Gcg<sup>fl/fl</sup> and Gcg<sup>gut−/−</sup> mice. (C, D) Time course of total tGLP-1 levels during metformin stimulus and corresponding AUC analysis in Gcg<sup>fl/fl</sup> and Gcg<sup>gut−/−</sup> mice. (E, F) Time course of blood glucose levels during metformin stimulus and corresponding AUC analysis in Gcg<sup>fl/fl</sup> and Gcg<sup>gut−/−</sup> mice. (G, H) Time course of total tGLP-1 levels during metformin stimulus and corresponding AUC analysis in Gcg<sup>fl/fl</sup> and Gcg<sup>gut−/−</sup> mice. Gcg<sup>fl/fl</sup>, n = 19 (males); Gcg<sup>gut−/−</sup>, n = 11 (males); Gcg<sup>fl/fl</sup>, n = 35 (males + females); Gcg<sup>gut−/−</sup>, n = 13 (males + females). Statistical significance was determined using the two-tailed t-test. ***P < 0.001; ****P < 0.0001.

In summary, the current findings highlight the importance of the gastrointestinal tract, and particularly the distal gut, as key sites of Gcg expression linked to the acute stimulation of L cell secretion and increased levels of circulating GLP-1 in mice. Nevertheless, these studies have limitations that restrict conclusions to the experimental models employed herein. It must be noted that rapid pharmacological parenteral administration of secretagogues would reach the distal gut and its relatively greater mass of L cells much faster than oral gavage of nutrient-related L cell secretagogues preferentially targeting the smaller number of L cells in the jejunum. Hence, the interpretation of the data should be tempered by these differences in routes of administration and rates of access of various secretagogues to L cells distributed within the proximal and distal guts.

Notably, we studied lean, nonobese mice without high-fat feeding, obesity, or diabetes, conditions that might modify the nature and responsivity of L cells along the gastrointestinal tract [42]. Importantly, GIP is known to increase GLP-1 secretion in mice [10], and some of the secretagogues employed here, such as olive oil and AR231453, are also known to enhance GIP secretion. Moreover, plasma GIP levels are increased in mice following the reduction of gut Gcg expression [13]. Hence, it remains possible that, for some secretagogues, the extent of change in plasma GLP-1 levels might also be influenced by simultaneous enhancement of the GIP secretion.

Our analyses of plasma GLP-1 levels were limited to measurements at baseline and only 1 or 2 time points, precluding definitive assessment of any pattern in GLP-1 secretory responses that might become apparent with more frequent blood sampling. Furthermore, experimental obesity and diabetes are frequently associated with pancreatic islet inflammation and enhanced α-cell GLP-1 production [35], conditions absent in the lean healthy mice examined in our current studies. Moreover, our studies employed mice with germline elimination of Gcg expression within the small and large bowel; hence, it is possible that adaptive compensatory mechanisms arising during growth and development of the gut may have contributed to the pharmacological responses observed herein. Nevertheless, our results, together with recent studies linking selective colonic L cell activation to increased plasma levels of GLP-1 [43], clearly demonstrate that the
Distal gut represents an important site for robust L cell secretion, capable of impacting circulating levels of GLP-1. These findings have potential relevance for informing strategies targeting L cells for the treatment of metabolic disorders.

**AUTHORS’ CONTRIBUTIONS**

BP, BY, DM, JK, YS, and DD designed the experiments and, together with DS, reviewed and analyzed the data and wrote and/or reviewed the manuscript. BP, BY, DM, JK, and YS carried out the experiments. DJD secured funding for the studies and is the guarantor of the data.

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Figure 5: Gut Ggc expression and increases in plasma GLP-1 are not required for metformin-mediated glucoregulation. Oral glucose tolerance tests (oGTT, 2 g/kg BW) and intraperitoneal glucose tolerance tests (ipGTT, 1.5 g/kg BW) were conducted in adult GgcGut+/+, and littermate control mice. All mice were given water, metformin 50 mg/kg, or metformin 150 mg/kg by oral gavage 60 min prior to glucose bolus in a triple-crossover study design. (A, B) Glucose levels and AUC analysis from oGTT in GgcGut+/+ mice. (C) Insulin and (D) total GLP-1 (tGLP-1) levels during oGTT at 0, 15, and 60 min after glucose in GgcGut+/+ mice. (E, F) Glucose levels and AUC analysis from oGTT in GgcGut+/- mice. (G) Insulin and (H) tGLP-1 levels during oGTT at 0, 15, and 60 min after glucose in GgcGut+/- mice. (I, J) Glucose levels and AUC analysis from ipGTT in GgcGut+/+ mice. (K) Insulin and (L) tGLP-1 levels during ipGTT at 0, 15, and 60 min after glucose in GgcGut+/+ mice. (M, N) Glucose levels and AUC analysis from ipGTT in GgcGut+/- mice. (O) Insulin and (P) tGLP-1 levels during ipGTT at 0, 15, and 60 min after glucose in GgcGut+/- mice. GgcGut+/-, n = 17–22 (males); GgcGut+/-, n = 6–9 (males). Statistical significance was assessed using one-way ANOVA with Tukey’s multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
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CONFLICTS OF INTEREST

DJD receives consulting honoraria from Intarcia, Merck, Novo Nordisk, Pfizer, and Sanofi within the past 12 months for advisory boards and lectures related to incretin biology. None of the other authors have conflicts of interest. Following the completion of these studies, BP became a full-time employee of Roche Canada Inc.

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