Mechanisms Underlying Metformin-Induced Secretion of Glucagon-Like Peptide-1 from the Intestinal L Cell

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Glucagon-like peptide-17-36NH2 (GLP-1) is secreted by the intestinal L cell in response to both nutrient and neural stimulation, resulting in enhanced glucose-dependent insulin secretion. GLP-1 is therefore an attractive therapeutic for the treatment of type 2 diabetes. The antidiabetic drug, metformin, is known to increase circulating GLP-1 levels, although its mechanism of action is unknown. Direct effects of metformin (5–2000 μM) or another AMP kinase activator, aminimidazole carboxamide ribonucleotide (100–1000 μM) on GLP-1 secretion were assessed in murine human NCI-H716, and rat FRIC L cells. Neither agent stimulated GLP-1 secretion in any model, despite increasing AMP kinase phosphorylation (P < 0.05–0.01). Treatment of rats with metformin (300 mg/kg, per os) or aminimidazole carboxamide ribonucleotide (250 mg/kg, sc) increased plasma total GLP-1 over 2 h, reaching 37 ± 9 and 29 ± 9 pg/ml (P < 0.001), respectively, compared with basal (7 ± 1 pg/ml). Plasma activity of the GLP-1-degrading enzyme, dipeptidylpeptidase-IV, was not affected by metformin treatment. Pretreatment with the nonspecific muscarinic antagonist, atropine (1 mg/kg, iv), decreased metformin-induced GLP-1 secretion by 55 ± 11% (P < 0.05). Pretreatment with the muscarinic (M) 3 receptor antagonist, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (500 μg/kg, iv), also decreased the GLP-1 area under curve, by 48 ± 8% (P < 0.05), whereas the antagonists pirenzepine (M1) and gallamine (M2) had no effect. Furthermore, chronic bilateral subdiaphragmatic vagotomy decreased basal secretion compared with sham-operated animals (7 ± 1 vs. 13 ± 1 pg/ml, P < 0.001) but did not alter the GLP-1 response to metformin. In contrast, pretreatment with the gastrin-releasing peptide antagonist, RC-3095 (100 μg/kg, sc), reduced the GLP-1 response to metformin, by 55 ± 6% (P < 0.01) at 30 min. These studies elucidate the mechanism underlying metformin-induced GLP-1 secretion and highlight the benefits of using metformin with dipeptidylpeptidase-IV inhibitors in patients with type 2 diabetes. (Endocrinology 152: 4610–4619, 2011)
endogenous GLP-1 has been heightened (13–15). GLP-1 secretion occurs primarily in response to nutrient ingestion (16), through a complex array of direct and indirect mechanisms. Postprandial GLP-1 levels peak within 15–30 min of nutrient consumption (17, 18). However, this response cannot be explained by direct nutrient stimulation of the L cell, because the majority of the L cells in the human and rodent intestine are localized in the distal gut (19), and nutrients do not normally transit to this region within this timeframe (20). Consistent with these observations, we have demonstrated the presence of an indirect loop that mediates this early phase of secretion, whereby nutrients entering the proximal gut stimulate the release of GLP-1 from L cells in the distal gut. In rodents, this pathway is mediated through activation of the vagus nerve (21) by the other incretin hormone, glucose-dependent insulinotropic peptide (GIP) (21, 22). This indirect pathway is further mediated by the enteric neuropeptide, gastrin-releasing peptide (GRP) (23, 24), and the muscarinic (M) 1 receptor (25). Similarly, administration of either a cholecystokinin receptor antagonist or of the general muscarinic antagonist, atropine, reduces nutrient-induced GLP-1 secretion in healthy subjects, indicating the relevance of this proximal-distal loop in humans (26, 27). Finally, passage of nutrients and, particularly, fats to the distal ileum enhances GLP-1 release through direct interactions with the intestinal L cell (28–31).

The biguanide, metformin, has been used as a treatment for T2DM for over 50 yr and, combined with lifestyle and diet change, has become the first-line therapy for the disease (32). Metformin reduces hyperglycemia through decreased hepatic glucose output and enhanced glucose uptake by skeletal muscle, at least in part through an AMP kinase (AMPK)-dependent mechanism (33–35). However, metformin also exerts AMPK-independent effects, including sensitization of the β-cell to GLP-1 and GIP through peroxisome proliferator-activated receptor α (36). Excitingly, in addition to acting as an incretin sensitizer, metformin has also been shown to enhance plasma levels of GLP-1. Although Molloy et al. (37) first demonstrated a link between metformin therapy and enhanced plasma glucagon-like immunoreactivity in normal individuals, Mannucci et al. (38, 39) reported that metformin increases plasma concentrations of active GLP-1 in obese, nondiabetic, as well as in obese, diabetic subjects. It was initially proposed that inhibition of DPP-IV could account for the increase in circulating levels of the active peptide (38, 40–42), although these findings are controversial (36, 43, 44). Furthermore, an in vitro study by Hinke et al. (45) revealed that metformin does not directly inhibit DPP-IV activity, and Yasuda et al. (43) demonstrated that acute administration of metformin or other biguanides to DPP-IV-deficient rats markedly enhances plasma bioactive GLP-1 levels. Consistent with these findings, Migoya et al. (44) recently reported that a 2-d pretreatment of healthy subjects with metformin enhances levels of total (active plus inactive forms) but not active GLP-1 after a meal, whereas coadministration of metformin and sitagliptin increases both active and total levels. Moreover, metformin has no effect on levels of GIP, which is also inactivated by DPP-IV, in humans or mice (36, 44), further suggesting that metformin does not increase GLP-1 levels through effects on DPP-IV. Although these studies provide a compelling argument for a role of metformin as a GLP-1 secretagogue, the mechanism by which metformin enhances GLP-1 release remains unknown. The purpose of the current study was therefore to determine whether metformin exerts direct effects on the intestinal L cell as a GLP-1 secretagogue and, if not, to assess the indirect mechanism(s) underlying the acute effects of metformin to increase plasma levels of GLP-1 in vivo.

Materials and Methods

In vitro studies

Cell cultures

Fetal rat intestinal cell (FRIC) cultures were prepared as previously described (46–48). In brief, intestinal cells from 19- to 21-d fetal Wistar rats were enzymatically dispersed, plated at a density of 0.6 intestines per 60-mm dish in culture medium [DMEM: 5% fetal bovine serum (FBS), 4.5 g/liter glucose, 40 U/ml penicillin, and 40 μg/ml streptomycin], and incubated overnight at 37 C and 5% CO2 with constant humidity. Animal procedures were approved by the Animal Care Committee of the University of Toronto.

Murine GLUTag and human NCI-H716 cells were propagated, as previously described (49–51), in culture media [DMEM (GLUTag) or RPMI (NCI-H716; American Type Culture Collection, Manassas, VA), with 5% FBS, and 4.5 g/liter glucose] on 10-cm dishes. Two days before experimental use, cells were seeded in 24- or six-well plates coated with poly-D lysine (GLUTag) or Matrigel (NCI-H716) for secretion or Western blot studies, respectively.

Secretion experiments

On the day of the experiment, cells were washed twice with Hanks’ balanced salt solution (HBSS) and incubated for 2 h with DMEM containing 0.5% FBS, 1 g/liter glucose, and 10 μg/ml insulin (FRIC), DMEM plus 0.5% FBS (GLUTag), or RPMI plus 0.5% FBS (NCI-H716) alone (negative control), or media plus forskolin (adenyl cyclase activator) and 3-isobutyryl-1-methylxanthine (IBMX) (phosphodiesterase inhibitor; 1 or 10 μM each; positive control; Sigma Chemical Co., St. Louis, MO), metformin (5–2000 μM; Sigma Chemical Co.), or aminimidazole carboxamide ribonucleotide (AICAR) (AMPK activator; 100–1000 μM; Toronto Research Chemicals, Toronto, Ontario, Canada). For metformin-sensitization experiments,
GLUTag cells were washed with HBSS, preincubated with or without metformin (2000 μM) in DMEM plus 0.5% FBS for 1 h, washed with HBSS, and incubated for an additional 2 h with forskolin and IBMX (10 μM each), bethanechol (muscarinic agonist, 500-1000 μM; Sigma Chemical Co.), bethanechol (500–1000 μM) plus metformin (2000 μM), or metformin alone (2000 μM). The timing of the metformin pretreatment was selected based on previous studies demonstrating that metformin increases plasma GLP-1 levels in rats within 1 h of administration (43). After the incubation period for all experiments, cells were checked by microscopy to ensure there were no observable morphological differences between treatment groups. Media and cell peptides were purified by reversed-phase adsorption (C18 Sep-Pak; Waters, Milford, MA), as previously described (25, 52), and stored at −20 C until RIA for total GLP-1 using an anti-GLP-1(x-36NH2) antiserum (Enzo Life Sciences, Plymouth Meeting, PA). Percent GLP-1 secretion was calculated as the amount of GLP-1 in the media normalized to the total GLP-1 in the dish or well (i.e. medium plus cell content). The total content of GLP-1 in each dish or well was not affected by any of the treatments (data not shown).

Western blot analysis

GLUTag and NCI-H716 cells were washed with HBSS and incubated for 1 h at 37 C in DMEM plus 0.5% FBS (GLUTag) or RPMI plus 0.5% FBS (NCI-H716) alone (negative control), or supplemented with metformin (2000 μM) or AICAR (1000 μM), followed by Western blot analysis for AMPK phosphorylation. FRIC cultures were not analyzed by immunoblot, because the heterogeneous nature of the cells precludes identification of L cell-specific responses. Cells were then washed in HBSS, collected into cold RIPA buffer containing EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany), sonicated, and stored at −20 C until immunoblot. Total protein content was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated on 7.5% SDS-PAGE, transferred onto a polyvinylidifluoride membrane (Bio-Rad Laboratories), and incubated overnight with a rabbit antiphosphorylated-AMPK antiserum (1:1000; Cell Signaling, Beverly, MA), followed by visualization using a horseradish peroxidase-linked goat-antirabbit secondary antibody (1: 2000; Cell Signaling) and an electrochemical luminescence detection system (Amersham Pharmacia Biotech, Baie D’Urfe, Quebec, Canada). Membranes were then stripped and reprobed using a rabbit anti-AMPK antiserum (1:1000; Cell Signaling) and a rabbit antiactin antiserum (1:1000; Sigma Chemical Co.). Membranes were imaged using a Kodak Image Station 4000MM PRO system (Carestream Molecular Imaging, New Haven, CT).

In vivo studies

Animals

Male Wistar rats (250 – 350 g; Charles River Laboratories, St. Constant, Quebec, Canada) were housed with a standard 12-h light, 12-h dark cycle for at least 2 wk before the experiment and were handled daily for 3–5 d before the experiment to reduce stress. Animals were fed regular chow and fasted overnight (16 h) before each experiment. All animal procedures were approved by the Animal Care Committee of the University of Toronto.

Secretion experiments

Conscious rats were treated with metformin (300 mg/kg, per os), AICAR (250 mg/kg, sc), or an equal volume of vehicle (sterile saline, per os and sc). For the inhibitor studies, rats were administered atropine sulfate (general muscarinic antagonist; 1 mg/kg, iv; Sigma Chemical Co.), pirenzepine (M1 antagonist; 0.5 mg/kg, iv; Sigma Chemical Co.), gallamine (M2 antagonist; 0.5 mg/kg, iv; Sigma Chemical Co.), 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) (M3 antagonist; 0.5 mg/kg, iv; Tocris Chemical Co.), or an equal volume of vehicle (sterile saline, iv or sc, as appropriate), 30 min before treatment with metformin or vehicle, as previously. Blood samples (200 μl) were collected from the lateral saphenous vein into heparinized glass capillary tubes and immediately placed on ice. An anticoagulant and protease inhibitor solution [5000 KIU/ml aprotinin (Sigma Chemical Co.), 1 mM diprotin A (DPP-IV inhibitor; Sigma Chemical Co.), and 4.1 mM EDTA] was added to all samples, to a final concentration of 10% (vol/vol), except for those used for the DPP-IV activity assay, in which diprotin A was excluded. As a positive control for successful inhibition with atropine, pirenzepine or 4-DAMP, salivary secretion was collected for 1 min from the oral cavity, using a Q-tip, before and after administration of the drug.

Plasma total and active GLP-1 were assayed using a sandwich immunooassay kit from Meso Scale Discovery (Gaithersburg, MD). Changes in the levels of active GLP-1 were found to closely reflect those of total GLP-1 in response to metformin treatment (data not shown). However, because active GLP-1 levels were not detectable unless the animals were also pretreated with sitagliptin (DPP-IV inhibitor; 5 mg/kg, iv; Merck Canada, Kirkland, Quebec, Canada), all further analyses were conducted using the total GLP-1 kit.

Plasma DPP-IV activity was measured using a colorimetric assay (Sigma Chemical Co.). One unit is defined as the production of 1.0 μmole of 4-nitroaniline from Gly-Pro p-nitroanilide per minute.

Bilateral subdiaphragmatic vagotomy

After a laparoscopic incision in anesthetized rats, the stomach was retracted caudally to expose the esophagus and the anterior and posterior branches of the vagus nerve. A small section (0.5 cm) of each branch was resected to vagotomize animals, whereas the nerves were visualized but not cut in sham-operated animals. The muscular and cutaneous incisions were closed with absorbable and silk suture, respectively, and animals were allowed to recover for at least 1 wk before being subjected to secretion experiments, as previously. Body weight was monitored daily; no abnormal (i.e. >20%) decreases in weight were observed.

Data Analysis

All data are expressed as the mean ± sem. In vitro GLP-1 secretion is expressed as a percentage of control secretion. AMPK phosphorylation is expressed as the fold of control. In vivo GLP-1 concentrations are expressed either as absolute values or as the change (i.e. ∆) from basal levels. ∆ Area under curve (AUC) analysis was calculated using the trapezoidal rule. In some experiments, data were converted to log10 to normalize variance for statistical analysis. Statistical analyses were performed using Statistical Analysis System software (SAS Institute, Cary, NC).
Differences between experimental groups were determined by Student’s t test or one- or two-way ANOVA, as appropriate, followed by n-1 post hoc comparisons, as appropriate. Significance was assumed at \( P < 0.05 \).

**Results**

**Metformin increases plasma GLP-1 levels *in vivo***

To confirm the actions of metformin *in vivo*, rats were treated with metformin, AICAR, or vehicle, and plasma levels of total GLP-1 were determined over 2 h (Fig. 1A). Orally administered metformin significantly increased total GLP-1 concentrations at 60 and 120 min (to 212 ± 73%, \( P < 0.05 \); and 771 ± 168%, \( P < 0.001 \), of vehicle controls, respectively). Subcutaneous administration of the AMPK agonist, AICAR, similarly increased total GLP-1 levels at 60 and 120 min (to 519 ± 75%, \( P < 0.001 \); and 354 ± 96%, \( P < 0.05 \), of vehicle controls, respectively). Because metformin treatment did not affect plasma DPP-IV activity (Fig. 1B), these findings suggested that the effect of metformin on plasma GLP-1 concentrations *in vivo* is mediated through increased peptide secretion rather than via decreased degradation.

**Metformin does not increase GLP-1 secretion *in vitro***

To establish whether metformin acts directly on the intestinal L cell to stimulate GLP-1 secretion, rat FRIC, murine GLUTag, and human NCI-H716 cultures were incubated with concentrations of metformin that ranged from clinically relevant (5–15 \( \mu M \)) (53, 54) to suprapharmacologic (45–2000 \( \mu M \)). Cells were also incubated with AICAR as a positive control for AMPK stimulation, as well as with forskolin plus IBMX as a cell function control. Neither metformin nor AICAR stimulated GLP-1 secretion, compared with vehicle, in any of the three L cell models (Fig. 2, A–C). In contrast, treatment with forskolin and IBMX increased GLP-1 secretion, to 4.4 ± 0.7-, 4.2 ± 1.7-, and 2.5 ± 0.3-fold of control, in FRIC, GLUTag, and NCI-H716 cells, respectively (\( P < 0.001 \)). Furthermore, incubation of the cells with very high concentrations of metformin (2000 \( \mu M \)) or AICAR (1000 \( \mu M \)) increased AMPK phosphorylation in GLUTag cells (to 1.7 ± 0.2-fold, \( P < 0.01 \) and 1.2 ± 0.2-fold, \( P < 0.05 \) of control, respectively), as well as in NCI-H716 cells (to 2.1 ± 0.3-fold, \( P < 0.01 \) and 1.5 ± 0.2-fold, \( P < 0.05 \) of control, respectively).

To determine whether metformin increases L cell sensitization to a known L cell secretagogue, bethanechol (25, 55), GLUTag cells were pretreated with or without metformin, followed by treatment with secretagogues in the absence or continued presence of metformin (Fig. 2D). As previously, forskolin plus IBMX induced a robust increase in GLP-1 secretion, compared with control cells preincubated with vehicle, and this response was not affected by pre- or coincubation with metformin. Bethanechol treatment (1000 \( \mu M \)) also increased GLP-1 secretion, to 2.6 ± 0.4-fold of control (\( P < 0.001 \)) in cells preincubated with vehicle. However, again, pre- or coincubation with metformin did not alter the L cell response to bethanechol. Furthermore, pre- or cotreatment with metformin did not alter GLP-1 secretion in the presence of a suboptimal concentration of bethanechol (500 \( \mu M \)). Collectively, therefore, the results of the *in vitro* studies indicated that metformin does not stimulate GLP-1 secretion through direct effects on the intestinal L cell.
Metformin-induced GLP-1 secretion \textit{in vivo} is M3 muscarinic receptor but not vagus dependent

To elucidate whether the stimulatory effects of metformin on GLP-1 secretion \textit{in vivo} involved the parasympathetic nervous system, a known regulator of the intestinal L cell (21, 25, 26, 55), rats were pretreated with the general muscarinic receptor antagonist, atropine, or with antagonists for each of the three muscarinic receptors previously demonstrated to be expressed by the rat L cell; pirenzepine, gallamine, and 4-DAMP, for the M1, M2, and M3 muscarinic receptors, respectively (25, 55). None of the antagonists alone caused changes in circulating levels of GLP-1 (Fig. 3). In contrast, atropine significantly decreased metformin-induced GLP-1 secretion at 30, 60, and 90 min (by 81 ± 11%, \(P < 0.01\); 68 ± 18%, \(P < 0.05\); and 42 ± 14%, \(P < 0.05\), respectively). The \(\Delta\) AUC was thus decreased by 55 ± 11% \((P < 0.05)\) in these animals compared with those treated with vehicle plus metformin alone (Fig. 3E). Pretreatment with pirenzepine or gallamine had no effect on either GLP-1 secretion or the \(\Delta\) AUC (Fig. 3, B, C, and E). In contrast, pretreatment with 4-DAMP resulted in a significant reduction in GLP-1 secretion at 30 and 60 min (to 60 ± 12%, \(P < 0.05\); and 62 ± 3%, \(P < 0.05\), respectively), and the \(\Delta\) AUC was correspondingly reduced, by 48 ± 8%, \((P < 0.05)\) (Fig. 3, D and E). Successful blockade of the muscarinic receptors was
verified by significant reductions in salivary secretion after pretreatment of rats with atropine, pirenzepine, and 4-DAMP (data not shown).

To investigate the involvement of the vagus nerve in mediating metformin-induced GLP-1 secretion, rats were subjected to a bilateral subdiaphragmatic vagotomy. Body weights decreased by the same extent in both groups of animals for up to 1 wk after surgery, and there was no difference between vagotomized and sham-operated animals on the day of the experiment (427 ± 9 and 428 ± 11 g, respectively). Vagotomized animals demonstrated a significant decrease in basal levels of total GLP-1 (by 38 ± 6%, P < 0.05), consistent with the known role of the vagus in stimulating GLP-1 secretion (21). However, GLP-1 release in response to metformin treatment was not different between vagotomized rats and sham-operated animals (Fig. 3F). The results of these studies therefore implicate nonvagal M3 muscarinic pathways in the regulation of metformin-induced GLP-1 secretion.

Metformin-induced GLP-1 secretion is partially dependent upon the GRP receptor

Finally, a role for the enteric neuropeptide, GRP, was evaluated, because GRP has been demonstrated to be a potent GLP-1 secretagogue in vitro and in vivo (23, 50, 56, 57). Pretreatment with the GRP receptor antagonist, RC3095, produced a brief, but significant, decrease in metformin-induced GLP-1 secretion (to 52 ± 6%, P < 0.05, compared with vehicle plus metformin) at 30 min, resulting in a 66 ± 15% (P < 0.05) reduction in the AUC at this time point (Fig. 4).
metformin.

plus metformin; 4616 Mulherin was 1.1 after oral gavage. Basal secretion for the vehicle plus metformin group (os). A second injection of the inhibitor, or vehicle, was given 30 min before treatment with metformin (300 mg/kg, per os) or vehicle (per os). A second injection of the inhibitor, or vehicle, was given 30 min after oral gavage. Basal secretion for the vehicle plus metformin group was 1.1 ± 0.4 pg/ml and was not significantly different from basal secretion in animals pretreated with antagonist. Diamonds, Vehicle plus metformin; squares, inhibitor plus metformin; circles, inhibitor plus vehicle. n = 6; *, P < 0.05 compared with vehicle plus metformin.

FIG. 4. A Total GLP-1 plasma levels in rats pretreated with the GRP receptor antagonist, RC-3095 (100 μg/kg, sc), or vehicle (sc), 30 min before treatment with metformin (300 mg/kg, per os) or vehicle (per os). A second injection of the inhibitor, or vehicle, was given 30 min after oral gavage. Basal secretion for the vehicle plus metformin group was 1.1 ± 0.4 pg/ml and was not significantly different from basal secretion in animals pretreated with antagonist. Diamonds, Vehicle plus metformin; squares, inhibitor plus metformin; circles, inhibitor plus vehicle. n = 6; *, P < 0.05 compared with vehicle plus metformin.

The exact mechanism by which these effects are exerted has remained unexplored. The results of the present study indicate that metformin stimulates GLP-1 release through a mechanism that involves both muscarinic (M3) and GRP receptor-dependent pathways but that is independent of both DPP-IV and direct effects on the intestinal L cell.

Three in vitro models of the intestinal L cell were employed to determine whether metformin directly stimulates GLP-1 secretion, at concentrations ranging from clinically relevant to suprapharmacologic (53, 54). The FRIC cultures are a heterogeneous population of primary FRIC (13). In contrast, the murine GLUTag cells are a homogeneous immortalized L cell line derived from a mouse expressing a proglucagon promoter, simian virus 40 large T-antigen transgene (14), whereas the human NCI-H716 cells originated from a spontaneous human cecal adenocarcinoma (15). These models, representing both primary and immortal L cells, as well as three different species, have been demonstrated to secrete GLP-1 in a regulated manner in response to a wide variety of known nutritional, hormonal, and neural secretagogues (16–18). However, treatment of these L cell models with either metformin or AICAR had no direct effect on GLP-1 secretion, although very high concentrations of both agents increased AMPK phosphorylation in the GLUTag and NCI-H716 cells. Notwithstanding, a recent study has suggested a role for AMPK in mediating berberine-induced GLP-1 secretion in the NCI-H716 cells (19). Berberine is a plant-derived isoquinoline alkaloid that has been demonstrated to have hypoglycemic effects in association with enhanced GLP-1 release in vitro but that is effective as an L cell secretagogue in vitro only at concentrations that are 10,000 times greater than those achieved by in vivo treatment. These findings therefore suggest that although activation of AMPK in the L cell can enhance GLP-1 secretion in vitro under some conditions, this pathway is unlikely to have any relevance in the physiologic or clinical setting.

Metformin has well-established actions as an insulin and leptin sensitizer, in addition to its more recent characterization as an incretin sensitizer in the β-cell (36, 59, 60). Therefore, we also investigated the effects of metformin as an L cell sensitizer in vitro, using the same timeframe (i.e. 1 h) for pretreatment as was found to be required for metformin to enhance plasma GLP-1 concentrations in vivo. However, preincubation of GLUTag cells with metformin did not enhance GLP-1 release in response to the general muscarinic agonist and known L cell secretagogue, bethanechol (25, 55), nor did it enhance the effects of subthreshold concentrations of bethanechol. Furthermore, metformin pretreatment also had no effect on the response to the cAMP-dependent pathway of GLP-1 secretion. Collectively, therefore, our in vitro studies suggest that metformin does not act directly on the L cell to induce GLP-1 secretion or enhance L cell sensitivity to several known secretagogues.

Previous studies have shown that metformin inhibits DPP-IV activity in vivo, in humans and rodents, leading to the suggestion that metformin enhances active GLP-1 concentrations through a DPP-IV-dependent mechanism (38, 40–42). However, our data indicated that metformin increases plasma total GLP-1, which is a better indicator of GLP-1 secretion than levels of active GLP-1, because it is independent of changes in DPP-IV activity. Of note, we also observed that metformin increases circulating levels of active GLP-1, although only in rats that were pretreated with sitagliptin to inhibit DPP-IV activity before metformin administration, further suggesting that the actions of metformin are DPP-IV independent. Importantly, these findings are consistent with the results of Yasuda et al. (43), who demonstrated that metformin increases plasma active GLP-1 levels in DPP-IV-deficient rats. Although we cannot dismiss the possibility that metformin affects the activity of the anchored form of DPP-IV, localized on the surface of epithelial tissues, including the capillaries in close proximity to the L cell (61), our findings with total GLP-1 argue against any actions of metformin to enhance GLP-1 levels through effects on DPP-IV.

To determine which indirect pathways might mediate metformin-induced GLP-1 secretion, we examined several mediators previously reported to regulate the indirect effects of nutrients on the intestinal L cell, specifically the muscarinic-, vagal-, and GRP-dependent pathways. Interestingly, our data demonstrated that both atropine and the
M3 receptor antagonist, 4-DAMP, but not the M1 and M2 receptor antagonists, reduced metformin-induced GLP-1 secretion in vivo, albeit only a single dose each of metformin and the inhibitors was tested. However, the comparable inhibition profiles for atropine and 4-DAMP suggest that the M3 receptor may be the primary muscarinic receptor responsible for mediating the effect of metformin on GLP-1. Anini et al. (25) previously reported expression of the muscarinic receptor subtypes M1-M3 by the rat intestinal L cell in vivo. However, the results of that study demonstrated a role for only the M1 receptor in nutrient-induced stimulation of GLP-1 secretion. Furthermore, the M3 receptor was shown to be inactive in both the rat and human L cell in vitro (25, 55). Notably, despite this role for the M3 receptor, the effects of metformin were found to be independent of the vagus nerve, although basal GLP-1 levels were reduced in vagotomized animals, consistent with known role for the vagus in regulating GLP-1 secretion (21). Hence, these findings suggest that the M3-dependent effects of metformin are mediated indirectly, rather than directly through a receptor that is expressed by the L cell. Additionally, as the enteric neuropeptide, GRP, has been demonstrated to play a role in nutrient-induced GLP-1 secretion (23, 24), this pathway was also evaluated as a potential mediator of metformin-induced GLP-1 secretion. Administration of the GRP receptor antagonist, RC-3095, reduced the effects of metformin briefly but significantly. RC-3095 is known to have a very short half-life in vivo (62), which may account for the transient nature of the effects. Finally, although atropine sulfate is known to cross the blood-brain barrier (63), neither 4-DAMP nor RC-3095 do so (64, 65). Together, these findings suggest that at least part of the actions of metformin on GLP-1 secretion must be exerted through a peripheral M3 receptor and GRPergic pathway.

It is recognized that the metformin-induced increases in GLP-1 may have occurred through decreased renal clearance of the peptide. However, because the rat kidney does not appear to express M3 muscarinic receptors (64), this possibility seems unlikely given the ability of 4-DAMP to block the actions of metformin on GLP-1. Similarly, given that bile acids are potent GLP-1 secretagogues (30), it has recently been suggested that metformin increases GLP-1 secretion by inhibiting carrier-mediated uptake of bile acids in the intestine (66). However, as the present study was conducted in fasted rats, the effects of metformin in these animals was presumably independent of bile acids. One final possibility is that metformin may increase mRNA levels for the GLP-1 prohormone, proglucagon, as recently reported by others for murine large intestine, 1 h after administration of metformin (44). However, the dose of metformin used in that study was higher than in the present study (500 vs. 300 mg/kg). Furthermore, the total content of GLP-1 in our in vitro studies was not altered by 2–3 h of incubation with metformin, suggesting that translation to active peptide does not occur within this timeframe, at least in our models. Finally, as recently reported by others for mice (36), we did find that acute administration of AICAR to rats increased plasma levels of GLP-1. These findings suggest a role for AMPK in mediating metformin-induced effects on GLP-1 release. How these findings with AICAR relate to the roles of the M3 and GRP receptors in the actions of metformin remains to be determined.

Although the present study has elucidated the mechanism of action of metformin to increase GLP-1 secretion in the fasted rat under acute conditions, it is recognized that this does not truly reflect the clinical setting, in which patients are normally exposed to metformin for prolonged periods of time with regular food intake. Notwithstanding these differences, chronic treatment of humans with metformin enhances both basal and nutrient-induced GLP-1 secretion (38, 39, 44), although acute administration has not been demonstrated to increase circulating levels of GLP-1 (39–41). These discrepancies are most likely due to differences in the dose of metformin used in human vs. rodent studies. Future studies should therefore investigate the mechanisms involved in the enhancement of GLP-1 secretion that are induced by chronic metformin treatment.

Collectively, the results of the present study indicate that a dose of 300 mg/kg metformin enhances circulating levels of GLP-1 in rats through a peripheral M3 and GRP receptor-dependent mechanism. These findings reinforce clinical reports of improved glycemic control in subjects treated with metformin plus DPP-IV inhibitors, which appear to be consequent to the additive effects of increased GLP-1 release and decreased GLP-1 degradation (44, 67). Further elucidation of the mechanisms underlying metformin-induced GLP-1 secretion will not only provide a more comprehensive understanding of the benefits of co-administration of metformin and incretin therapy but also a more thorough understanding of the pathways regulating GLP-1 secretion.

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