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Ghrelin Is a Novel Regulator of GLP-1 Secretion

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GLP-1 is a gastrointestinal L-cell hormone that enhances glucose-stimulated insulin secretion. Hence, strategies that prevent GLP-1 degradation or activate the GLP-1 receptor are used to treat patients with type 2 diabetes. GLP-1 secretion occurs after a meal and is partly regulated by other circulating hormones. Ghrelin is a stomach-derived hormone that plays a key role in whole-body energy metabolism. Because ghrelin levels peak immediately before mealtimes, we hypothesized that ghrelin plays a role in priming the intestinal L-cell for nutrient-induced GLP-1 release. The intraperitoneal injection of ghrelin into mice 15 min before the administration of oral glucose enhanced glucose-stimulated GLP-1 release and improved glucose tolerance, whereas the ghrelin receptor antagonist D-Lys GHRP-6 reduced plasma levels of GLP-1 and insulin and diminished oral glucose tolerance. The ghrelin-mediated improvement in glucose tolerance was lost in mice coinjected with a GLP-1 receptor antagonist as well as in *Glp1r*^{-/-} mice lacking the GLP-1 receptor. The impaired oral glucose tolerance in diet-induced obese mice was also improved by ghrelin preadministration. Importantly, ghrelin directly stimulated GLP-1 release from L-cell lines (murine GLUTag, human NCI-H716) through an extracellular signal-related kinase 1/2-dependent pathway. These studies demonstrate a novel role for ghrelin in enhancing the GLP-1 secretory response to ingested nutrients.

GLP-1 is a gastrointestinal hormone secreted from the enteroendocrine L-cell in response to nutrient ingestion. Once released into the circulation, GLP-1 elicits a potentiation of glucose-stimulated insulin secretion from the β -cells within the pancreatic islets, known as the incretin effect (1,2). The actions of incretin hormones, including

GLP-1 as well as glucose-dependent insulinotropic peptide (GIP), on insulin secretion result in improved glucose clearance, and as such, incretin-based approaches are an important therapeutic tool in the treatment of patients with type 2 diabetes mellitus (T2DM). Current incretin therapies include long-acting GLP-1 receptor (GLP-1R) agonists and also inhibitors of incretin hormone degradation; however, GLP-1 secretagogues represent a potential third approach to enhancing incretin action in T2DM (1–3).

GLP-1 secretion is regulated by a combination of nutrient-, neural-, and hormonal-activated pathways. Although nutrients have been shown to directly enhance GLP-1 release from the intestinal L-cell (4,5), the enteric and parasympathetic nervous systems (6,7) and other endocrine hormones are likely more critical mediators of the very rapid effect of meal ingestion on circulating levels of GLP-1. Several examples of the hormonal regulation of GLP-1 have been demonstrated. GIP enhances GLP-1 secretion from the rodent L-cell in vivo (7) and in vitro (8,9), whereas cholecystokinin appears to be more important in humans (10). The satiety factor, leptin, also stimulates GLP-1 release by rodent and human L-cells (11), as does the metabolic hormone, insulin (12). Interestingly, several studies have indicated that L-cell responses can also be “primed,” such that preexposure to one secretagogue modulates the subsequent response to a heterologous effector. Thus, pretreatment of the murine intestinal GLUTag L-cell line with the nutrient oleic acid enhances the subsequent GLP-1 secretory response to GIP (13). Conversely, prolonged exposure to leptin and insulin suppresses the GLP-1 secretory response to oral glucose administration in vivo in rodents (11,12).

One hormone that has not been investigated with respect to effects on the L-cell is the stomach-derived,

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acylated peptide, ghrelin. Plasma ghrelin levels, like those of GLP-1, fluctuate in response to nutrient ingestion. However, in contrast to GLP-1, ghrelin levels are highest immediately before habitual mealtimes (14). Although ghrelin is best known for its appetite-inducing effects (15), several groups have also demonstrated a role for ghrelin in enhancing disposition of ingested calories (16,17). Hence, ghrelin promotes the synthesis of fatty acids and triglycerides in the liver (18) as well as their storage within human adipocytes (19). These actions suggest a role for ghrelin in preparing the body for an incoming meal. Because GLP-1 enhances the insulin response necessary for the disposition of these ingested nutrients, we hypothesized that the L-cell response to meal ingestion may be “primed” by the high levels of ghrelin that occur immediately before a meal. Consistent with this notion, premeal ghrelin levels (20,21) and the GLP-1 response to a meal (22) are concurrently reduced in obese individuals. Thus, ghrelin treatment may play a role in enhancing GLP-1 secretion in this condition.

The current study examined the effects of acylated ghrelin pretreatment on GLP-1 secretion and associated glucose tolerance in response to oral glucose administration in normal mice, mice with blockade of the ghrelin receptor and genetic disruption of GLP-1R signaling, and mice with diet-induced obesity. To determine the mechanism of action of ghrelin on the L-cell, murine and human L-cell models were used to also examine its effects in vitro on GLP-1 release and downstream signaling. Collectively, the results indicate that preexposure to ghrelin enhances GLP-1 release in response to a subsequent oral glucose load and that this effect is mediated through a ghrelin receptor- and extracellular signal-related kinase (ERK)1/2-dependent pathway.

RESEARCH DESIGN AND METHODS

Animal Studies

Male C57BL/6 mice (4 or 7 weeks old) were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Male GLP-1R wild-type (WT) and knockout (*Glp1r*^{-/-}) mice on a C57BL/6 background (10–12 weeks old) have been described previously (23). The University of Toronto and Mount Sinai Hospital Animal Care Committees approved all animal work.

Mice were fasted overnight (16 h) before receiving an oral gavage of 5 g/kg D-glucose in water (preliminary studies indicated that a 16-h fast enabled detection of a greater GLP-1 response than found after a 6-h fast; data not shown). Treatments or vehicle (sterile 0.9% NaCl) were administered intraperitoneally 15 min before the glucose gavage. All peptides were obtained from Bachem Americas Inc. (Torrance, CA). Blood samples (1 drop) for glucose measurements were obtained at 0, 10, 60, 90, and 120 min after oral glucose loading and were analyzed using a OneTouch Ultra 2 glucose meter (LifeScan Canada, Ltd., Burnaby, British Columbia, Canada). Additional blood samples (100 μ L) were collected in EDTA-coated capillary

tubes (Sarstedt, Montreal, Quebec, Canada) at 0, 10, and 60 min after oral glucose administration and were immediately spiked with 10 μ L of a concentrated protease inhibitor solution containing aprotinin (5,000 KIU), diprotin A (0.1 mmol/L), and EDTA (1.2 mg/mL) and kept on ice until centrifugation at 4,000g for 5 min and collection of plasma. Total GLP-1 and insulin concentrations were determined using kits from Meso Scale Discovery (Rockville, MD). Plasma samples for fasting ghrelin were collected without the addition of the protease inhibitor solution and were analyzed using an unacylated ghrelin ELISA kit (Cayman Chemical, Ann Arbor, MI).

To induce weight gain and glucose intolerance, 4-week-old mice were fed a high-fat diet (HFD; 60% calories from fat; Research Diets, New Brunswick, NJ) for 10 weeks. Because preliminary tests showed that these mice were unable to rapidly clear a glucose load of 5 g/kg (i.e., glucose levels were higher than the limit of glucometer detection of 33 mmol/L during an oral glucose tolerance test [OGTT]; data not shown), a lower dose of 2 g/kg was used for these mice.

In Vitro Studies

L-Cell Models

Mouse GLUTag cells were originally derived by single-cell cloning of a proglucagon-SV40 large T antigen-induced tumor (24). Cells were maintained in high-glucose (25 mmol/L) DMEM supplemented with 10% FBS and were passaged after trypsinization. Human NCI-H716 cells, derived from a cecal tumor, were obtained from American Type Culture Collection (Manassas, VA) and maintained in suspension culture in RPMI medium with 11 mmol/L glucose and supplemented with 10% FBS and penicillin/streptomycin. Both cells lines have been extensively validated as models for GLP-1 secretion by the intestinal L-cell (4,5,11,12,25).

GLP-1 Secretion Assay

GLUTag cells were split into 24-well plates at a density of 200,000 cells per well in 1 mL culture medium. NCI-H716 cells plated at the same cell density required that cell culture plates be precoated with 0.5 mg/mL Matrigel basement membrane solution in Hank's Balanced Salt Solution (HBSS) for 1 h to allow attachment. Cells were rinsed with HBSS 48 h after plating and treated in the corresponding culture medium with reduced serum (0.5% FBS). Some GLUTag cells were treated in media containing 0–5 mmol/L glucose. After a 2-h incubation, media were collected and acidified (final concentration 0.1% trifluoroacetic acid), and cells were collected in acid lysis buffer (1% trifluoroacetic acid, 1 N HCl, 5% formic acid, and 0.1 mmol/L NaCl). Peptides were collected by reversed-phase adsorption to C18 silica (Sep-Pak, Waters, Ontario, Canada), and samples were dried in vacuo before radioimmunoassay using a total GLP-1 kit (Millipore Canada Ltd.).

Western Blot

GLUTag cells were split into 12-well plates at 500,000 cells per well. After 2 days in culture, cells were rinsed in

HBSS and incubated in HBSS containing 5.5 mmol/L glucose for 1 h under standard culture conditions. Experiments where inhibitors were used received an additional 30-min preincubation of vehicle, U-0126 (10 μ mol/L), or PD98059 (10 μ mol/L). Cells were then incubated for 10 min in HBSS containing various treatments. After this, cells were lysed and collected in radioimmunoprecipitation assay cell lysis buffer supplemented with PhosphoStop and Complete Mini (EDTA-free) enzyme inhibitors (Roche Diagnostics, Indianapolis, IN). Proteins were quantified using the Bradford method, and protein (50 μ g) was loaded onto 8% acrylamide SDS-PAGE Tris-glycine gels. Proteins were transferred to polyvinylidene fluoride membranes, blocked with 5% skim milk in Tris-buffered saline, and then incubated with primary antibodies (phospho and total ERK1/2; Cell Signaling Technologies, Danvers, MA) overnight in 5% BSA. Proteins were visualized using appropriate horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell Signaling Technologies) and were imaged and quantitated using Luminata Forte chemiluminescence substrate (Millipore) with a Kodak digital imaging system.

RT-PCR

GLUTag and NCI-H716 cells were seeded into 10-cm plates at 2,000,000 cells per plate. After 2 days in culture, RNA was harvested using the Qiagen RNeasy total RNA extraction kit with QIAshredder (Qiagen, Toronto, Ontario, Canada). Total RNA (2 μ g) was converted to cDNA using 5 \times All-in-One RT MasterMix (Applied Biological Materials, Richmond, British Columbia, Canada). Primers for the ghrelin receptor were ordered from IDT (Coralville, IA): human forward: GTGAAAATGCTGGCTGTAGTGG, and reverse: TGATGGCAGCACTGAGGTAG; and mouse forward: GTCTCATCGGGAGGAAGCTG and reverse: AGCGCTGAGGTAGAAGAGGA. Mouse brain cDNA was used as a positive control. PCR products were run and visualized on a 1% agarose gel.

Statistical Analyses

All data are expressed as the mean \pm SE. Studies comparing two groups were analyzed by Student *t* test. All animal experiments where comparisons were made between two independent variables (i.e., time and drug treatment) were analyzed by two-way ANOVA, followed by Bonferroni post hoc tests at individual time points where applicable. Vertical parentheses with # symbols to the right of the graphs indicate significant interaction, and * over individual time points indicate significance at that time point for the post hoc test. Area under the curve (AUC) analysis was calculated using the trapezoidal method and was assessed by Student *t* test or one-way ANOVA, followed by Bonferroni post hoc tests where applicable. The relative insulin response was calculated as $\Delta\text{Insulin}_{\text{AUC}(0-60\text{min})} / \Delta\text{glucose}_{\text{AUC}(0-60\text{min})}$. Cell culture data were expressed as percent GLP-1 secretion [media content/(media + cell content)] normalized to vehicle control. Cell culture experiments in which multiple doses of ghrelin were tested were analyzed by one-way ANOVA, followed by a Bonferroni post hoc test.

Group and post hoc effects with a *P* value of <0.05 were considered significant.

RESULTS

Ghrelin Pretreatment Enhances Glucose-Stimulated GLP-1 Secretion and Glucose Homeostasis

To determine the effect of ghrelin pretreatment on GLP-1 secretion, mice were injected with acylated ghrelin (200 nmol/kg) or vehicle at $t = -15$ min, and blood was sampled before ($t = 0$ min) and during an OGTT. Ghrelin administration had no effect on baseline GLP-1 levels (Fig. 1A); however, 10 min after oral glucose loading, ghrelin-pretreated mice had significantly higher levels of circulating GLP-1 than mice given vehicle alone ($P < 0.001$, Fig. 1A). Although insulin levels were not significantly increased by ghrelin pretreatment (Fig. 1B), the relative insulin response was elevated in response to ghrelin ($P < 0.05$) in association with significantly improved glucose tolerance ($P < 0.05$ at $t = 60$ min and $P < 0.01$ at $t = 90$ min, Fig. 1C and D).

Ghrelin Receptor Antagonism Impairs GLP-1 Secretion and Glucose Homeostasis

To determine the importance of endogenous ghrelin in GLP-1 secretion and glucose homeostasis, mice were pretreated with the ghrelin receptor antagonist, D-Lys GHRP-6 (10 μ mol/kg), or vehicle alone, 15 min before an OGTT. Blocking the ghrelin receptor caused a significant reduction in glucose-stimulated levels of circulating GLP-1 (Fig. 2A). These mice also had a significant decrease in the insulin response to oral glucose compared with saline controls 10 min after oral glucose administration ($P < 0.01$ at $t = 10$ min and $P < 0.05$ for the effects of ghrelin compared with saline over the entire time course; Fig. 2B), as well as impaired clearance of the oral glucose load ($P < 0.001$ at $t = 60$ min, $P < 0.01$ at $t = 90$ min, and $P < 0.01$ overall by two-way ANOVA; Fig. 2C).

The GLP-1R Is Required for Ghrelin's Effects on Glucose Metabolism

To establish that the effects of ghrelin on oral glucose handling were mediated through the GLP-1R pathway, mice were pretreated with ghrelin (200 nmol/kg) and the GLP-1R antagonist exendin-4⁹⁻³⁹ (5 nmol/kg) or with vehicle and exendin-4⁹⁻³⁹ alone. The stimulatory effect of ghrelin pretreatment on GLP-1 secretion during an OGTT was maintained in the presence of GLP-1R antagonism ($P < 0.01$ vs. vehicle alone; Fig. 3A). However, the ability of the ghrelin injection to subsequently enhance insulin secretion and glucose tolerance was completely lost in mice cotreated with exendin-4⁹⁻³⁹ (Fig. 3B and C).

To further validate the importance of the GLP-1 pathway in mediating the effects of ghrelin on glucose tolerance, GLP-1 levels were assessed in *Glp1r*^{-/-} mice. Similar to the responses observed in C57BL/6 mice, WT mice demonstrated a significant increase in the GLP-1 response ($P < 0.01$ at $t = 10$ min and $P < 0.05$ overall by two-way ANOVA; Fig. 4A) and an improvement in glucose tolerance ($P < 0.05$; Fig. 4B) when injected 15 min before an OGTT with

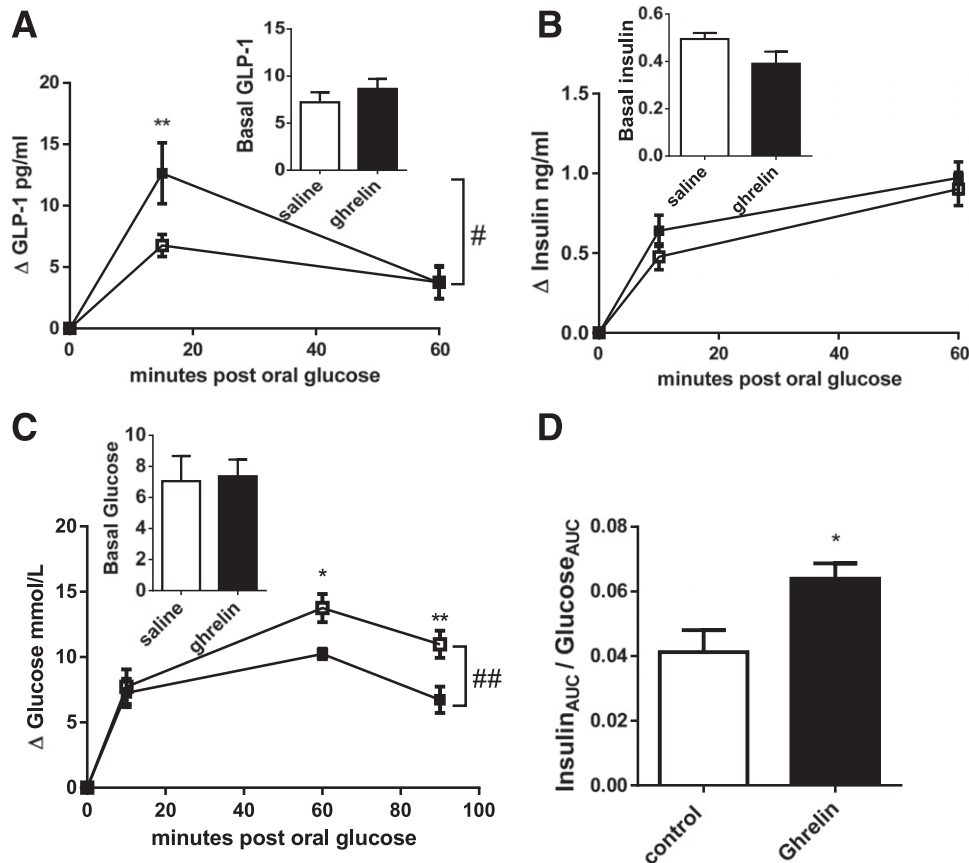


Figure 1—Ghrelin pretreatment enhances glucose-stimulated GLP-1 secretion. Fasted mice were injected with 200 nmol/kg ghrelin (■) or saline (□) 15 min before oral glucose administration and determination of plasma GLP-1 (A), plasma insulin (B), and blood glucose (C) at 0, 10, and 60 min (insets: fasting levels). D: AUC of insulin relative to AUC of glucose. Data are presented as mean \pm SE, $n = 6$ –10. * $P < 0.05$, ** $P < 0.01$ at individual time points; # $P < 0.05$, ## $P < 0.01$ for overall group effect in a two-factor ANOVA.

ghrelin (200 nmol/kg) compared with vehicle alone. Interestingly, fasting levels of GLP-1 were extremely elevated in *Glp1r*^{-/-} mice (160 pg/mL, data not shown), consistent with previous reports that demonstrated elevated levels of plasma exendin-4 after exogenous administration in studies of *Glp1r*^{-/-} mice (26). However, the ghrelin-induced improvement in glucose tolerance was completely abrogated in *Glp1r*^{-/-} mice (Fig. 4C).

Ghrelin Administration Restores GLP-1 Responses and Glucose Tolerance in Obese Mice

Because obesity is often associated with decreased fasting ghrelin levels and impaired glucose tolerance, we next determined whether ghrelin pretreatment of obese mice could improve oral glucose tolerance in association with enhanced GLP-1 secretion. After 10 weeks of being fed the HFD, mice had a significant increase in body weight compared with paired chow-fed controls (42.6 ± 1.9 vs. 26.8 ± 1.2 g, $P < 0.5$). Obese mice had significantly lower pre-OGTT levels of ghrelin (Fig. 5A). During the OGTT, obese mice demonstrated significantly impaired glucose tolerance compared with control mice ($P < 0.01$ at $t = 60$ min, $P < 0.05$ at $t = 90$ min, and $P < 0.05$ overall; Fig. 5B). However, when obese mice were pretreated with ghrelin

(100 nmol/kg), glucose tolerance was restored to a level similar to that of chow-fed controls (Fig. 5B). This improvement in glucose tolerance was associated with a significant increase in GLP-1 levels compared with obese mice treated with vehicle alone ($P < 0.01$ at $t = 10$ min and $P < 0.05$ overall; Fig. 5C).

Ghrelin Stimulates GLP-1 Secretion From Murine and Human L-Cells

Finally, to determine whether the effects of ghrelin on the L-cell are exerted directly, GLP-1 secretion was examined in two different L-cell models: the mouse GLUTag and human NCI-H716 cell lines. Ghrelin receptor mRNA expression was detected in both cell lines by RT-PCR of total RNA (Fig. 6A). Both cell lines also demonstrated significant increases in GLP-1 secretion in response to treatment with varying doses of acylated ghrelin (1–100 nmol/L) in regular culture media ($P < 0.05$ at 1 and 10 nmol/L and $P < 0.01$ at 100 nmol/L for GLUTag cell, and $P < 0.05$ at 1 nmol/L and $P < 0.01$ at 10 nmol/L for NCI-H716 cells; Fig. 6B and C). To investigate the role of glucose in ghrelin-stimulated GLP-1 secretion, GLUTag cells were also examined at 0, 1, and 5 mmol/L glucose. Cells incubated in 0 and 1 mmol/L glucose did not

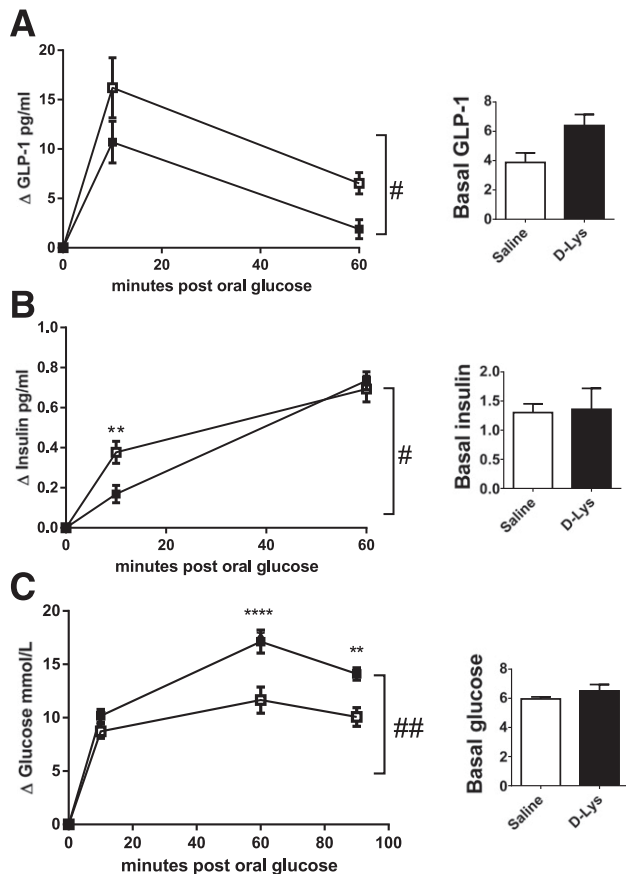


Figure 2—Ghrelin receptor antagonism impairs glucose-stimulated GLP-1 secretion. Fasted mice were injected with 10 μ mol/kg D-Lys GHRP-6 (■) or saline (□) 15 min before oral glucose administration and determination of plasma GLP-1 (A), plasma insulin (B), and blood glucose (C) at 0, 10, and 60 min (right panels: fasting levels). Data are presented as mean \pm SE, $n = 9$ –12. ** $P < 0.01$, **** $P < 0.0001$ at individual time points; # $P < 0.05$, ## $P < 0.01$ for overall group effect in a two-factor ANOVA.

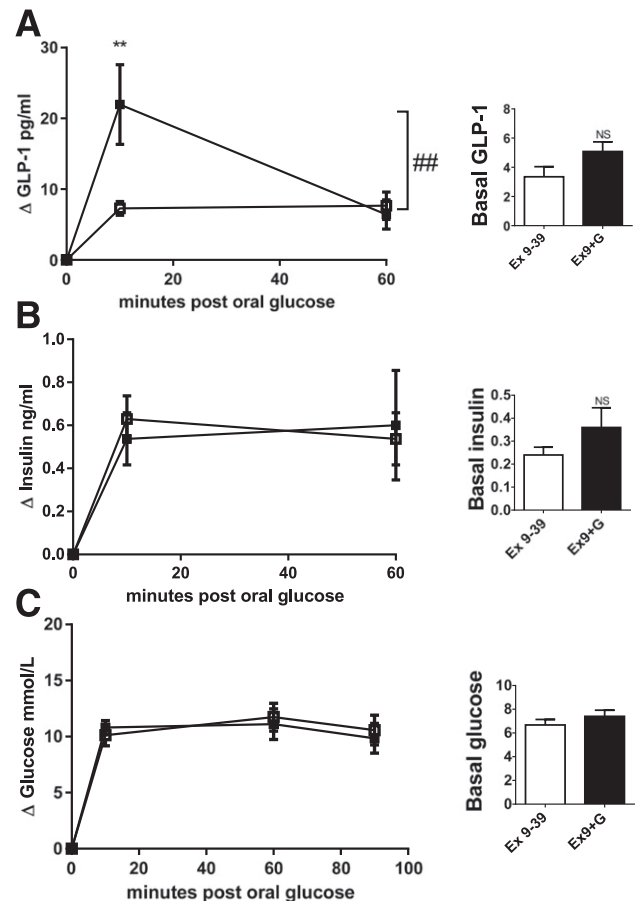


Figure 3—GLP-1R blockade prevents ghrelin's effects on glycemia. Fasted mice were injected with 50 nmol/kg exendin-4^{9–39} with (■) and without (□) 200 μ mol/kg ghrelin 15 min before oral glucose administration and determination of plasma GLP-1 (A), plasma insulin (B), and blood glucose (C) at 0, 10, and 60 min (right panels: fasting levels). Data are presented as mean \pm SE, $n = 9$. ** $P < 0.01$ at individual time points; ## $P < 0.01$ for overall group effect in a two-factor ANOVA; NS, not significant.

respond to ghrelin, whereas ghrelin significantly stimulated GLP-1 release in the presence of 5 mmol/L glucose (1.4 ± 0.2 -fold, $P < 0.05$).

To investigate the signaling pathway involved in ghrelin-stimulated GLP-1 release, GLUTag cells were treated with ghrelin for 10 min, followed by immunoblot for phospho- and total ERK1/2. Ghrelin induced a significant increase in the levels of phospho-ERK1/2 ($P < 0.05$), an effect that was completely blocked by pretreatment with the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (MEK)/ERK inhibitors U-0126 (Fig. 6D) and PD98059 (data not shown). Finally, to confirm a role for the MAPK pathway in ghrelin-mediated GLP-1 secretion, GLUTag cells were preincubated with U-0126 before being treated with ghrelin. ERK1/2 inhibition completely blocked the ability of ghrelin to stimulate GLP-1 secretion (Fig. 6E).

DISCUSSION

GLP-1 secretion has been shown previously to be regulated by orally ingested nutrients and by metabolic hormones,

including GIP, leptin, and insulin (4,7–13,24,25). Importantly, the L-cell response to these heterologous secretagogues appears to be integrated, such that pretreatment with one effector can modulate the subsequent response to another, thereby ensuring appropriate levels of circulating GLP-1 (11–13). Because ghrelin plays key roles in communicating whole-body energy status and its levels are highest before a meal, a time point that also immediately precedes the GLP-1 response to the ingested nutrients, we aimed to determine if ghrelin pretreatment could affect GLP-1 release in response to oral glucose. Administration of acylated ghrelin alone, in the absence of an oral glucose load, did not modulate basal levels of GLP-1; the effects of ghrelin on GLP-1 were only observed after oral glucose administration. Interestingly, the effects of insulin on GLP-1 release also require the presence of glucose (12). This requirement for glucose in the effects of ghrelin on the L-cell, an effect that was confirmed in vitro, is of particular significance because ghrelin levels are highest during fasting,

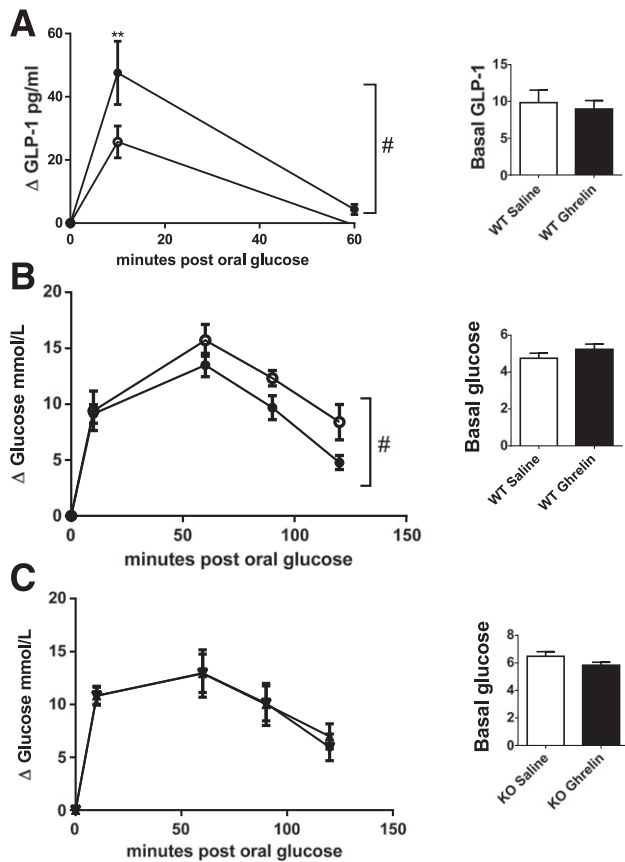


Figure 4—Mice lacking GLP-1R do not demonstrate ghrelin-induced improvements in glucose tolerance. Fasted WT mice were injected with 200 μ mol/kg ghrelin (closed symbols) or saline (open symbols) 15 min before oral glucose administration and determination of plasma GLP-1 (A) and blood glucose (B) at 0, 10, 60, and 90 min. C: Fasted *Glp1r*^{-/-} mice (knockout [KO]) were injected with 200 μ mol/kg ghrelin or saline 15 min before oral glucose administration and determination of blood glucose at 0, 10, 60, and 90 min. Right panels indicate fasting levels. Data are presented as mean \pm SE, $n = 4$. ** $P < 0.01$ at individual time points; # $P < 0.05$ for overall group effect in a two-factor ANOVA.

a period when the insulinotropic effects of GLP-1 are not required. Our findings therefore indicate that ghrelin alone does not lead to increases in GLP-1 but, rather, induces “priming” of the L-cell in preparation for subsequent oral glucose-stimulated GLP-1 secretion. Interestingly, the concept of priming has been established for other endocrine cells, including the effects of acetylcholine, γ -aminobutyric acid, and fatty acids on subsequent insulin release by the β -cell (27–29), as well as of a novel modulatory factor that modulates gonadotrope secretion of luteinizing hormone (30).

The results of the current study demonstrate that priming of the L-cell by ghrelin results in an improvement in oral glucose tolerance in lean and obese mice and that this is mediated through a direct mechanism of action in murine and human in vitro L-cell models. The physiological relevance of these findings in the context of the biological activities of GLP-1 is twofold. First, a small,

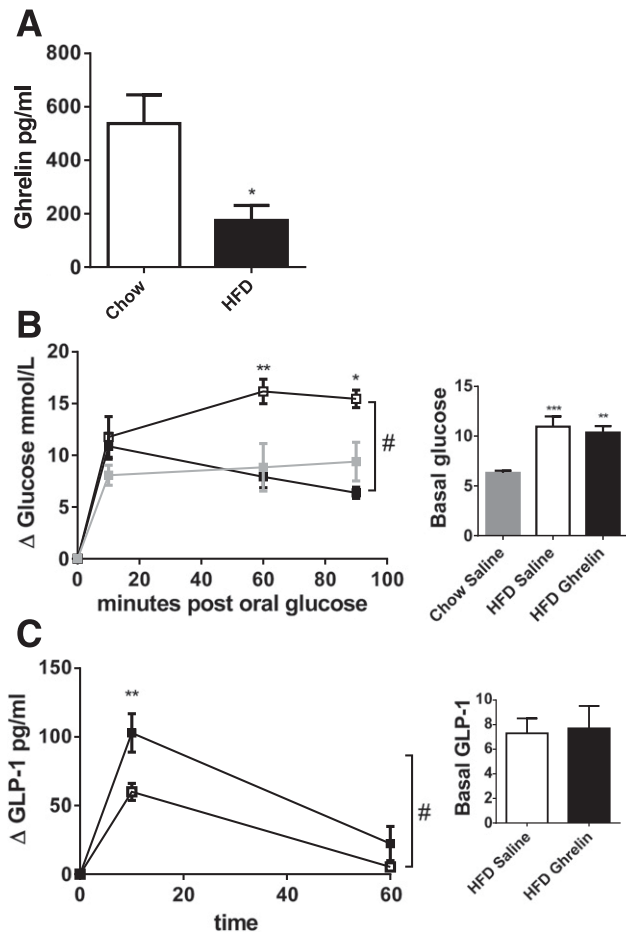


Figure 5—Ghrelin treatment in obese mice restores glucose tolerance and enhances GLP-1 secretion. A: Fasting ghrelin levels in chow-fed and HFD-fed mice. B: Glucose response curve to oral glucose in chow-fed (gray symbols), HFD-fed (open symbols), and HFD-fed/ghrelin-treated (closed symbols) mice (right panel: fasting levels). Statistical comparisons were made only between the HFD saline and HFD diet ghrelin groups. C: GLP-1 responses to oral glucose in HFD mice treated with saline (\square) or ghrelin (\blacksquare) (right panel: fasting levels). Data are presented as mean \pm SE, $n = 6–9$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. chow-fed or at individual time points; # $P < 0.05$ for overall group effect in a two-factor ANOVA.

albeit nonsignificant, increase in insulin levels was found in normal mice after ghrelin treatment before the OGTT. Because this occurred in the setting of reduced blood glucose levels, insulin levels relative to prevailing glycemia were therefore increased. This finding was complemented by the demonstration that insulin levels were decreased by pretreatment with a ghrelin receptor antagonist. Hence, some of the observed actions of GLP-1 to modulate glucose tolerance may have been modulated through the canonical incretin axis. However, an additional mechanism of action of GLP-1 also includes suppression of glucagon release (1,2), which may therefore have contributed to the glucose-lowering effects in our model.

Although the ghrelin doses used in the in vivo experiments are comparable with other studies examining

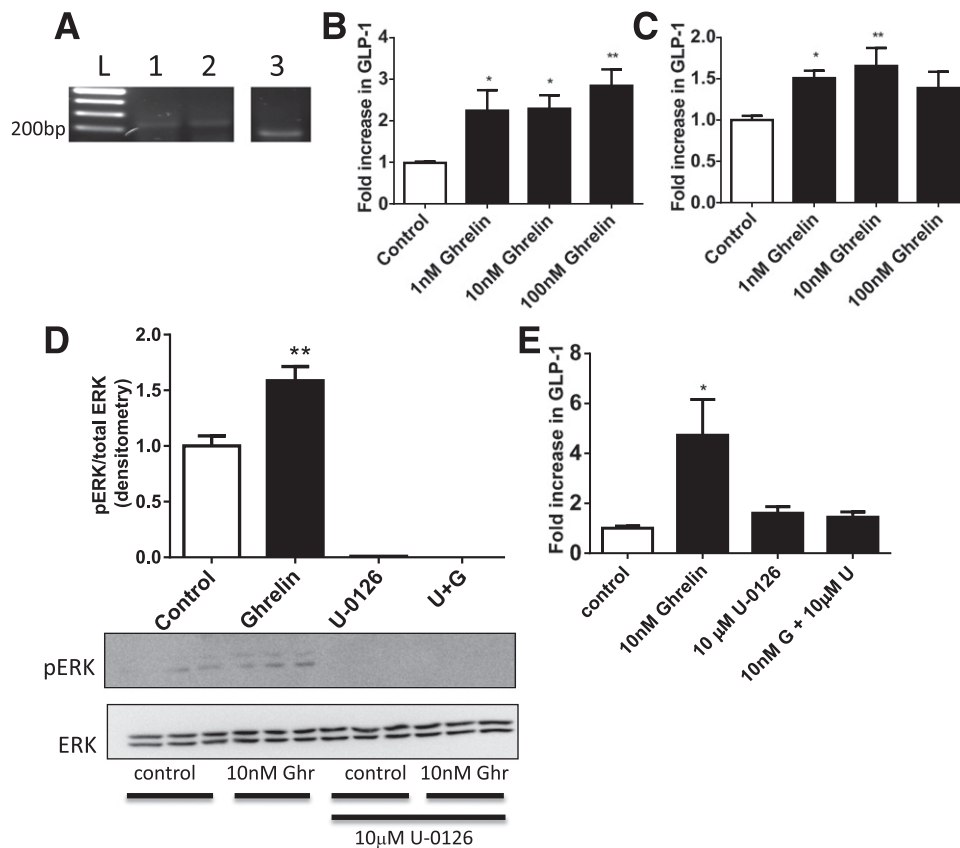


Figure 6—Ghrelin directly stimulates GLP-1 secretion from the L-cell. *A*: Agarose gel of RT-PCR products for the mouse ghrelin receptor (predicted size = 276 bp) in whole brain (*lane 1*) and GLUTag cells (*lane 2*) and the human ghrelin receptor (predicted size = 172 bp) in NCI-H716 cells (*lane 3*); molecular weight ladder (*L*). Ghrelin treatment stimulates GLP-1 secretion from GLUTag (*B*) and NCI-H716 (*C*) cells. *D*: Western blot and densitometry of phospho-(p)ERK1/2 and total ERK1/2 in GLUTag cells treated with 10 nmol/L ghrelin, with and without U-0126, for 30 min (a representative blot is shown; $n = 3$). *E*: GLP-1 secretion in cells pretreated with and without U-0126 and then treated with 10 nmol/L ghrelin. Data are presented as mean \pm SE, $n = 6$. * $P < 0.05$, ** $P < 0.01$ vs. control.

ghrelin action in vivo (31,32), they do not represent physiological concentrations of preprandial ghrelin. To resolve this issue, we used a ghrelin receptor antagonist-based approach to determine the role of endogenous ghrelin in L-cell secretory responses. Treatment of mice with the established ghrelin receptor antagonist, D-Lys GHRP-6, resulted in a decline in oral glucose-stimulated GLP-1 and insulin secretion as well as impaired glucose tolerance. These findings support our initial findings of a positive role for ghrelin in GLP-1 release and downstream glucose tolerance but differ from those of Dezaki et al. (33), who reported that D-Lys GHRP-6 coinjected with IP glucose caused an enhanced insulin response and an improvement in glucose clearance. In addition, a recent study by Tong et al. (34) demonstrated that an infusion of acylated ghrelin into normal humans not only increased fasting glucose levels but also decreased the disposition index (which measures β -cell function) during an intravenous glucose tolerance test. The discrepancy between the results of these studies and our findings on glucose tolerance likely lies in the different routes of glucose administration. The injection of glucose intraperitoneally or intravenously represents a significant difference from the more physiological oral route used in the

current study, in that only oral administration of glucose increases GLP-1 release (35,36), thereby inducing the incretin effect. Collectively, these findings suggest that ghrelin may play two distinct roles in the regulation of insulin and, in turn, glycemia. At the level of the β -cell, ghrelin can inhibit glucose-stimulated insulin secretion, a finding that is supported by in vitro β -cell experiments (37,38). However, as shown in our study, ghrelin also enhances oral glucose-induced GLP-1 release. The latter is likely of greater importance in the physiological regulation of glycemia because nutrients normally arrive through the oral route and the associated incretin response is responsible for most of the downstream effect on insulin by the β -cell (36).

Unexpectedly, somewhat variable total GLP-1 levels were found within and between the different strains of mice used in the current study. Hence, basal levels of GLP-1 in saline-injected controls ranged from 3 to 7 pg/mL in the C57BL/6 mice (Figs. 1–3), and this was increased slightly, to 10 pmol/L, in the GLP-1R WT mice (Fig. 4). The GLP-1 increments in response to identical OGTTs also varied somewhat, ranging from onefold (Fig. 1) to 2.5-fold (Figs. 2–4). However, the *Glp1r* animals were

studied in a separate animal facility from the normal C57BL/6 mice, and the GLP-1 data for each figure were also obtained using separate ELISA plates. Thus, whether these differences in GLP-1 levels are physiologically meaningful or simply represent normal experimental variation remains unclear.

Feeding mice the HFD for 8–10 weeks produced a marked elevation in body weight and a concomitant reduction in oral glucose tolerance. Interestingly, these mice also had significantly lower levels of fasting ghrelin, as reported previously (20,21). Although we did not observe an impairment in the GLP-1 response to oral glucose under this diet paradigm (data not shown), a longer state of obesity may lead to a blunted GLP-1 response, as is the case in chronic human obesity (21). Nevertheless, obese mice treated with ghrelin demonstrated a robust increase in their oral glucose-stimulated GLP-1 response and an improvement in glucose tolerance. These data suggest that in the state of obesity, lower circulating ghrelin levels may contribute to the impaired clearance of oral glucose. Whether these findings hold clinical relevance is unclear, because the administration of ghrelin is known to induce weight gain and adiposity in lean rodents (39,40). However, ghrelin administration to obese rodents has been shown to improve growth hormone responses without causing additional weight gain (41). Future long-term studies examining the chronic effects of ghrelin administration to obese mice on GLP-1 levels and glucose tolerance should resolve this issue.

To establish whether the effects of ghrelin on GLP-1 release *in vivo* could be mediated through direct actions on the intestinal L-cell, we examined well-established *in vitro* models of the murine and human L-cell. Ghrelin treatment of the murine GLUTag and human NCI-H716 cells stimulated GLP-1 secretion, an effect that was mediated through the MEK/ERK1/2 pathway. It is well established that the ghrelin receptor activates MEK/ERK signaling in a number of different cell types, including intestinal epithelial cells (42–44). Furthermore, the MEK/ERK pathway has been previously shown to be required for stimulation of GLP-1 secretion by meat hydrolysate (25) and insulin (12), with recent studies further implicating ERK1/2 signaling in the circadian regulation of GLP-1 release by multiple secretagogues (45). Although the precise mechanisms linking MEK/ERK activation to GLP-1 secretion requires further investigation, previous studies have demonstrated essential roles for rho guanosine 5'-triphosphatase, cell division cycle 42, p21-activated kinase-1, and remodeling of the cortical F-actin cytoskeleton in this pathway (46). In line with this, actin-interacting SNARE (SNAP [Soluble NSF Attachment Protein] Receptor) proteins are also expressed in L-cells and are required for the exocytosis of GLP-1 (47,48). Further molecular analyses of intestinal L-cell signaling and exocytosis will be required to elucidate the exact pathways underlying the priming of GLP-1 secretory responses.

In summary, we have demonstrated a role for ghrelin in the regulation of oral glucose-stimulated GLP-1 secretion

leading to an improvement in glucose clearance. Ghrelin actions are exerted directly on the L-cell and require the MAPK pathway. Finally, we have shown that ghrelin pretreatment of obese mice with reduced fasting ghrelin levels enhances GLP-1 secretion and restores glucose tolerance. Collectively, these findings indicate that ghrelin plays a novel role in the regulation of GLP-1 secretion.

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Ghrelin: A New Incretin Enhancer Therapy?



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The overarching goal of diabetes therapies is to get patients under good glycemic control using therapies that lower abnormally elevated plasma glucose, thereby reducing glucose toxicity, oxidative stress, and inflammation, conditions that promote cardiovascular disease (CVD) and chronic kidney disease. Unfortunately, some conventional diabetes therapies, although effective at controlling glycemia, induce weight gain and actually increase the risk of hypoglycemic episodes and associated CVD events. To this end, the development of novel, safe, and effective therapies that improve long-term glycemic control, that minimize the risk of hypoglycemia, that do not increase weight gain (or induce therapeutic weight loss), and that promote cardiovascular (CV) health is a better way forward (1). The ongoing development of incretin-enhancing therapies may be an example of such an approach. The gut-derived incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide, are secreted in response to the postprandial increase in glucose and act to augment glucose-dependent pancreatic insulin secretion, suppress glucagon release, slow gastric emptying, enhance satiety, and modulate the so-called gut-brain axis (2,3). The action of incretin hormones is short-lived due to rapid degradation by the ubiquitous enzyme, dipeptidyl peptidase-4 (DPP-4). Earlier studies showed that the incretin axis in individuals with type 2 diabetes mellitus (T2DM) is impaired (4,5), and this has led to the development of novel incretin-enhancing therapies, such as the stable (i.e., DPP-4-resistant) receptor (GLP-1R) agonists and DPP-4 inhibitors. Accumulating evidence suggests that modulation of incretin signaling by GLP-1R agonists or DPP-4 inhibitors is not only beneficial for improving glycemic control but confers neutral or modest CV protection (6,7). Given the complexity of the incretin axis and interaction with the gut-brain axis, it is likely that additional mechanisms, yet to be discovered, exist to enhance the incretin axis and improve glycemia in T2DM.

In this issue, Gagnon et al. (8) propose a novel mechanism to enhance the GLP-1 secretory response to ingested nutrients (Fig. 1A). The focus of their study is the stomach-derived hormone ghrelin, which is key to whole-body energy metabolism (9). Unlike GLP-1, which is secreted following nutrient ingestion, ghrelin levels are highest shortly before regular meal times. The premeal ghrelin surge suggested to the authors that ghrelin acts to prepare the body for the incoming meal. More specifically, it was hypothesized that the ghrelin surge may prime the intestinal L-cells to release GLP-1 in response to the upcoming meal. Given that obese individuals have reduced premeal ghrelin levels (10), it is possible that administration of exogenous ghrelin could serve to enhance GLP-1 secretion.

To test their hypothesis, Gagnon et al. first injected normal male C57BL/6 mice with acylated ghrelin or vehicle solution 15 min prior to performing an oral glucose tolerance test (OGTT). Blood samples were drawn before and after mice received the glucose load to evaluate pre- and postprandial circulating GLP-1, glucose, and insulin. Ghrelin pretreatment did not affect baseline GLP-1 level but lead to an almost twofold increase in GLP-1 levels 10 min after glucose loading compared with vehicle-treated mice. Glucose tolerance was significantly improved in response to ghrelin pretreatment, and this occurred in the absence of a significant effect on insulin levels. At this stage, the authors had their initial proof of concept that ghrelin pretreatment increased GLP-1 secretion and improved glucose handling in normal male mice.

Next, the investigative team tested whether ghrelin receptor antagonism could reduce glucose-stimulated GLP-1 secretion as well as glucose tolerance. The goal was the reverse of their first experiment (i.e., to establish the body's responses to endogenous rather than exogenous ghrelin secretion). In this protocol, mice were pretreated with the ghrelin receptor blocker, D-Lys GHRP-6,

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See accompanying article, p. 1513.

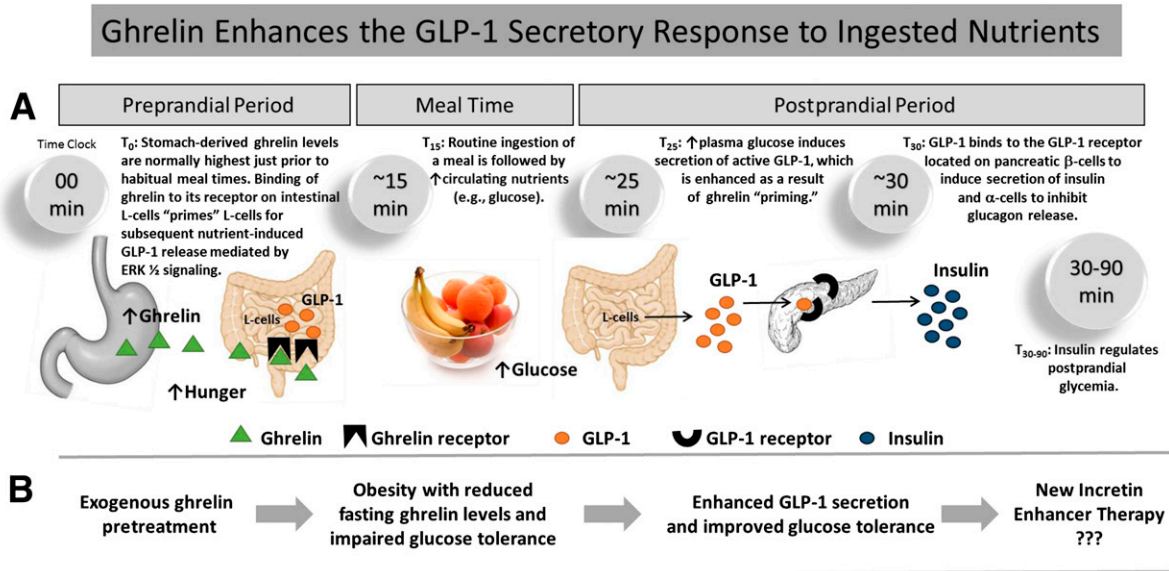


Figure 1—A: Proposed mechanism to explain a novel role for ghrelin in the regulation of oral glucose-stimulated GLP-1 secretion preprandial release of ghrelin that acts directly on ghrelin receptors located on enteroendocrine L-cells. This "priming" effect of ghrelin on nutrient-stimulated GLP-1 secretion requires signaling through the MAPK pathway. **B:** Obese mice pretreated with exogenous ghrelin exhibit enhanced GLP-1 secretion and normal glucose tolerance.

or a vehicle 15 min prior to OGTT. Compared with pretreatment with vehicle, ghrelin receptor blockade resulted in lower levels of circulating GLP-1. Moreover, the insulin response to glucose load was impaired as was the clearance of glucose. This experiment also provided proof of concept by demonstrating a role for endogenous ghrelin and the ghrelin receptor in the regulation of GLP-1 secretion and glucose handling.

Next, the authors tested whether the effects of ghrelin on glucose tolerance were mediated through the GLP-1R pathway. This experiment was important because we know that GLP-1 can exert effects through the GLP-1R, as well as through GLP-1R-independent pathways. Two protocols were performed to test this concept. The first involved simultaneous pretreatment of C57BL/6 mice with acylated ghrelin and the GLP-1R blocker, exendin-4, or a vehicle. In the second protocol, GLP-1R knockout mice were used in place of exendin-4. Similar to their first experiment described above, the authors demonstrated in these two protocols that ghrelin pretreatment stimulated secretion of GLP-1 during OGTT, despite the presence of the GLP-1R blocker. The downstream effects of exogenous ghrelin on insulin secretion and glucose handling during OGTT, however, were completely eliminated in the presence of exendin-4. These experiments provided proof of concept that GLP-1R is required for ghrelin's effects on glucose metabolism.

The final *in vivo* test of the hypothesis was designed to determine whether ghrelin pretreatment would sufficiently stimulate GLP-1 secretion to improve glucose handling in a model of diet-induced obesity caused by high-fat-diet feeding (Fig. 1B). Ten weeks of high-fat-diet feeding induced substantial weight gain, abnormally low

preprandial ghrelin levels, and impaired glucose tolerance, similar to what is observed in obese humans (10). Thus, the investigators used a preclinical model of obesity that has high translational relevance for testing the efficacy of novel therapies to treat the metabolic complications of obesity and diabetes. In the setting of obesity, ghrelin pretreatment normalized glucose tolerance, and this was likely due to increases in GLP-1 secretion and GLP-1R activation. Collectively, these *in vivo* results validate the hypothesis that ghrelin pretreatment induces GLP-1 secretion and improves glucose tolerance. Moreover, the hypothesis was validated in glucose-intolerant obese mice. These results will undoubtedly lead to further preclinical testing and are likely to be sufficient to warrant consideration of a phase one trial.

Finally, Gagnon et al. explored mechanisms to explain how preprandial ghrelin induces secretion of GLP-1. They verified expression of ghrelin receptor transcripts in both murine and human L-cell lines and demonstrated increases in GLP-1 secretion in response to the addition of acylated ghrelin to culture media, an effect that could be prevented by pharmacological blockade of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinases (ERK)1/2 activation. These *in vitro* studies indicate that ghrelin acts directly on L-cells via activation of the MAPK pathway. More details on mechanisms linking MEK/ERK activation to GLP-1 secretion are likely to emerge soon.

The results of the study by Gagnon et al. (8) suggest the potential for a third pharmacological strategy for enhancing the impaired incretin system in the obese population with diabetes. The authors have very elegantly identified ghrelin as a GLP-1 secretagogue. Prior to this

report, the only pharmacological agent or diabetes therapy identified as a GLP-1 secretagogue was metformin (11). Unlike ghrelin, metformin does not act directly on L-cells to induce GLP-1 release. Whether acylated ghrelin is ultimately developed as a therapy to correct diabetic dysglycemia remains to be seen. Like all newly developed therapies to treat diabetes, arduous work of rigorous testing will be required to determine its effects on body weight, risk for hypoglycemia, and CV health.

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