Ghrelin is a Novel Regulator of Glucagon-like Peptide-1 Secretion

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Abstract

Glucagon-like peptide-1 (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 regulated in part by other circulating hormones. Ghrelin is a stomach-derived hormone that plays a key role in whole-body energy metabolism. As ghrelin levels peak immediately before meal times, we hypothesized that ghrelin plays a role in priming the intestinal L-cell for nutrient-induced GLP-1 release. Intraperitoneal injection of ghrelin into mice 15 minutes before oral glucose administration 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 co-injected with a GLP-1 receptor antagonist as well as in *Glp1*r$^{-/-}$ mice lacking the GLP-1 receptor. The impaired oral glucose tolerance in diet-induced obese mice was also improved by ghrelin administration. Importantly, ghrelin directly stimulated GLP-1 release from L-cell lines (murine GLUTag, human NCI-H716) through an Erk1/2-dependent pathway. These studies demonstrate a novel role for ghrelin in enhancing the GLP-1 secretory response to ingested nutrients.

Introduction

Glucagon-like peptide-1 (GLP-1) is a gastrointestinal hormone secreted from the enteroendocrine L-cell in response to nutrient ingestion. Once released into 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 both long-acting GLP-1 receptor (GLP-1R) agonists and inhibitors of incretin hormone degradation; however, GLP-1 secretagogues represent a potential 3rd approach to enhancing incretin action in T2DM (1-3).

GLP-1 secretion is regulated by a combination of nutrient-, neural- and hormonal-activated pathways. While 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 pre-exposure to one secretagogue modulates the subsequent response to a heterologous effector. Thus, pre-treatment 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, 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 meal times (14). While 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. As 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, pre-meal 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 present study examined the effects of acylated-ghrelin pre-treatment on GLP-1 secretion and associated glucose tolerance in response to oral glucose administration in normal mice, mice with blockade of ghrelin and genetic disruption of GLP-1 receptor signaling, and mice with diet-induced obesity. To determine the mechanism of action of ghrelin on the L-cell, its effects on GLP-1 release and downstream signaling were also examined in vitro, utilizing both murine and human L-cell models. Collectively, the results indicate that pre-exposure 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 Erk1/2-dependent pathway.

Methods

Animal studies

Male C57BL/6 mice (7 wk old) were purchased from Charles River Laboratory (PQ, Canada). Male GLP-1R wild-type (WT) and knockout (*Glp1*r$^{-/-}$) mice on a C57Bl/6 background (10-12 wk old) have been described previously (23). All animal work was approved by the Animal Care Committees of the University of Toronto and Mount Sinai Hospital.

Mice were fasted overnight (16 hr) before receiving an oral gavage of 5g/kg D-glucose in water (preliminary studies indicated that a 16 hr fast enabled detection of a greater GLP-1 response than found following a 6 hr fast; data not shown). Fifteen min before the glucose gavage, treatments or vehicle (sterile 0.9% NaCl) were administered intraperitoneally (IP). 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 analysed using a One Touch Ultra2 glucose meter (Life Scan Canada, Ltd., Burnaby, BC, Canada). Additional blood samples (100ul) were collected in EDTA-coated capillary tubes (Sarstedt, Montreal, PQ, Canada) at 0, 10 and 60 min after oral glucose administration, and were immediately spiked with 10ul of a concentrated protease inhibitor solution containing Aprotinin (5000KIU), diprotin A (0.1mM) and EDTA (1.2mg/ml) and kept on ice until centrifugation at 4000 x g 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 analysed using an unacylated-ghrelin ELISA kit (Cayman Chemical, Ann Arbor MI).

To induce weight gain and glucose intolerance, 4-wk old mice were placed on a high-fat diet (60% calories from fat, HFD; Research Diets, New Brunswick, NJ) for 10 wk. As preliminary tests showed that these mice were unable to rapidly clear a 5g/kg glucose load (i.e. glucose levels were higher than the limit of glucometer detection (33 mM) during an OGTT; data not shown), a lower dose of 2g/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 tumour (24). Cells were maintained in high-glucose (25 mM) Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were passaged following 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 Roswell Park Memorial Institute (RPMI) medium with 11 mM 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 1ml of culture medium. NCI-H716 cells plated at the same cell density required that cell culture plates be pre-coated with 0.5mg/ml Matrigel® basement membrane solution (in Hank's Balanced Salt Solution (HBSS)) for 1 hr, in order to allow attachment. Forty-eight hours after

plating, cells were rinsed with HBSS and then treated in the corresponding culture medium with reduced serum (0.5% FBS). Some GLUTag cells were treated in media containing 0 – 5 mM glucose. Following a 2-hr incubation, media were collected and acidified (final concentration 0.1% trifluoroacetic acid) and cells were collected in acid lysis buffer (1% trifluoracetic acid, 1N HCl, 5% formic acid and 0.1mM NaCl). Peptides were then collected by reversed-phase adsorption to C18 silica (Sep-Pak, Waters, ON, Canada) and samples were dried in vacuum prior to 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 d in culture, cells were rinsed in HBSS then incubated in HBSS containing 5.5 mm glucose for 1 hour under standard culture conditions. Experiments were inhibitors were used received an additional 30 min pre-incubation of vehicle, U0126 (10μM), or PD98059 (10μM). Cells were then incubated for 10 min in HBSS containing various treatments. Following this, cells were lysed and collected in radio-immunoprecipitation 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 50ug of protein 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 Signalling Technologies, Danvers MA) overnight in 5% bovine serum albumin. Proteins were visualized using appropriate horseradish peroxidise 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 10cm plates at 2,000,000 cells per plate. After 2 d in culture, RNA was harvested using the Qiagen RNeasy total RNA extraction kit with QIAshredder (Qiagen, Toronto, ON, Canada). Total RNA (2µg) was converted to cDNA using 5x All-in-One RT MasterMix (Applied Biological Materials, Richmond, BC, 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 ± standard error. Studies comparing two groups were analyzed by Student's t test. All animal experiments where comparisons were made between two independent variables (i.e. time and drug treatment) were analyzed by 2-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. Asterisks 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's t test or 1-way ANOVA followed by Bonferroni post-hoc tests where applicable. The relative insulin response was calculated as $\Delta\text{Insulin}^{AUC(0\text{-}60min)} / \Delta\text{glucose}^{AUC(0\text{-}60min)}$. 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 less than 0.05 were considered significant.

Results

Ghrelin pre-treatment enhances glucose-stimulated GLP-1 secretion and glucose homeostasis

To determine the impact of ghrelin pre-treatment on GLP-1 secretion, mice were injected with acylated-ghrelin (200nmol/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 (Figure 1A). However, 10 min after oral glucose loading, ghrelin-pre-treated mice had significantly higher levels of circulating GLP-1 as compared to mice given vehicle alone $p<0.001$, Figure 1A). Although insulin levels were not significantly increased by ghrelin pre-treatment (Figure 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, Figure 1C-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 pre-treated with the ghrelin receptor antagonist, D-Lys GHRP-6 (10umol/kg) or vehicle alone, 15 min prior to an OGTT. Blocking the ghrelin receptor caused a significant reduction in glucose-stimulated levels of circulating GLP-1 (Figure 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 as compared to saline over the entire time course, Figure 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 2-way ANOVA, Figure 2C).

The GLP-1 receptor 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-1 receptor pathway, mice were pre-treated with both ghrelin (200nmol/kg) and the GLP-1 receptor antagonist exendin-4^{9-39} (5nmol/kg) or with vehicle and exendin-4^{9-39} alone. The stimulatory effect of ghrelin pre-treatment on GLP-1 secretion during an OGTT was maintained in the presence of GLP-1R antagonism ($p<0.01$ vs. vehicle alone, Figure 3A). However, the ability of ghrelin injection to subsequently enhance insulin secretion and glucose tolerance was completely lost in mice co-treated with exendin-4^{9-39} (Figure 3B-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 *Glp1*$r^{-/-}$ 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 2-way ANOVA, Figure 4A) and an improvement in glucose tolerance ($p<0.05$, Figure 4B) when injected with ghrelin (200nmol/kg) as compared to vehicle alone, 15 min prior to an OGTT. Interestingly, fasting levels of GLP-1 were extremely elevated in *Glp1*$r^{-/-}$mice (160pg/ml, data not shown), consistent with previous reports demonstrated elevated levels of plasma exendin-4 following exogenous administration in studies of *Glp1*$r^{-/-}$ mice (26). However, the ghrelin-induced improvement in glucose tolerance was completely abrogated in *Glp1*$r^{-/-}$ mice (Figure 4C).

Ghrelin administration restores GLP-1 responses and glucose tolerance in obese mice

As obesity is often associated with decreased fasting ghrelin levels, as well as impaired glucose tolerance, we next determined whether ghrelin pre-treatment of obese mice could improve oral glucose tolerance in association with enhanced GLP-1 secretion. After 10 weeks on a HFD, mice had a significant increase in body weight compared to 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 (Figure 5A). During the OGTT, obese mice demonstrated significantly impaired glucose tolerance as compared to control mice ($p<0.01$ at t=60 min, $p<0.05$ at t=90 min and $p<0.05$ overall, Figure 5B). However, when obese mice were pre-treated with ghrelin (100nmol/kg), glucose tolerance was restored to a level similar to that of chow-fed controls (Figure 5B). This improvement in glucose tolerance was associated with a significant increase in GLP-1 levels compared to obese mice treated with vehicle alone ($p<0.01$ at t=10 min and $p<0.05$ overall, Figure 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 2 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 (Figure 6A). Both cell lines also demonstrated significant increases in GLP-1 secretion in response to treatment with varying doses of acylated-ghrelin in regular culture media (1-100nM; $p<0.05$ at 1 and 10 nM, and $p<0.01$ at 100 nM for GLUTag cells; $p<0.05$ at 1 nM and $p<0.01$ at 10 nM for NCI-H716 cells, Figure 6B-C). To investigate the role of glucose in ghrelin-

stimulated GLP-1 secretion, GLUTag cells were also examined at 0, 1 and 5mM glucose. Cells incubated in 0 and 1mM glucose did not respond to ghrelin, whereas ghrelin significantly stimulated GLP-1 release in the presence of 5mM 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 10min 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 pre-treatment with the MEK/ERK inhibitors, U-0126 (Figure 6D) and PD98059 (data not shown). Finally to confirm a role for the MAPK pathway in ghrelin-mediated GLP-1 secretion, GLUTag cells were pre-incubated with U-0126 before being treated with ghrelin. ERK1/2 inhibition completely blocked the ability of ghrelin to stimulate GLP-1 secretion (Figure 6E).

Discussion

GLP-1 secretion has been shown previously to be regulated by both orally-ingested nutrients and 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 pre-treatment with one effector can modulate the subsequence response to another, thereby ensuring appropriate levels of circulating GLP-1 (11-13). As 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 pre-treatment 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 since ghrelin levels are highest during fasting, a period of time 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, GABA 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 present study demonstrate that priming of the L-cell by ghrelin results in an improvement in oral glucose tolerance in both lean and obese mice, and that this is

mediated through a direct mechanism of action in both 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 two-fold. First, a small albeit non-significant increase in insulin levels was found in normal mice following ghrelin treatment prior to the OGTT. As 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 pre-treatment 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.

While the doses of ghrelin used in the in vivo experiments are comparable to other studies examining ghrelin action in vivo (31; 32), they do not represent physiological concentrations of pre-prandial ghrelin. To resolve this issue, we utilized 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, who reported that D-Lys GHRP-6 co-injected with IP glucose caused an enhanced insulin response and an improvement in glucose clearance (33). In addition, a recent study by Tong et al. demonstrated that 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 (IV) glucose tolerance test (34). 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 either IP or IV represents a significant difference from the more physiological oral route utilized 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 since nutrients normally arrive through the oral route and the associated incretin response is responsible for a majority of the downstream effect on insulin by the β-cell (36).

Unexpectedly, somewhat variable total GLP-1 levels were found both within and between the different strains of mice utilized in the present study. Hence, basal levels of GLP-1 in saline-injected controls ranged from 3 – 7 pg/ml in the C57Bl/6 mice (Figures 1-3), and this was increased slightly, to 10 pM, in the GLP-1R WT mice (Figure 4). The GLP-1 increments in response to identical OGTTs also varied somewhat, ranging from 1-fold (Figure 1) to 2.5-fold (Figures 2, 3 and 4). However, it must be considered that the *Glp1*r 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 a 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). While 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 as well as 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, as 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 both 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 both 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 (CDC42), p21-activated kinase-1 (PAK1), and remodeling of the cortical F-actin cytoskeleton in this pathway (46). In line with this, actin-interacting SNARE 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 pre-treatment 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.

Author contributions J.G. researched data and wrote the manuscript; L.L.B. researched data and reviewed/edited the manuscript; D.J.D. reviewed/edited the manuscript; P.L.B. obtained funding, contributed to discussion and reviewed/edited the manuscript.

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Guarantor Dr. Patricia L. Brubaker is the guarantor of this work and, as such, had full access to all the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Figure Legends

Figure 1. Ghrelin pre-treatment enhances glucose-stimulated GLP-1 secretion. Fasted mice were injected with 200nmol/kg ghrelin (closed symbols) or saline (open symbols) 15 minutes before oral glucose administration and determination of (A) plasma GLP-1, (B) plasma insulin and (C) blood glucose at 0, 10 and 60 minutes (insets: fasting levels; open bar, saline; closed bar, ghrelin) D: AUC of insulin relative to AUC of glucose. Data are presented as mean +/- 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.

Figure 2. Ghrelin receptor antagonism impairs glucose-stimulated GLP-1 secretion. Fasted mice were injected with 10umol/kg D-Lys GHRP-6 (closed symbols) or saline (open symbols) 15 minutes before oral glucose administration and determination of (A) plasma GLP-1, (B) plasma insulin and (C) blood glucose at 0, 10 and 60 minutes (insets: fasting levels; open bar, saline; closed bar, D-Lys GHRP-6). Data are presented as mean +/- SE, n= 9-12. *$p<0.05$, **$p<0.01$, ***$p<0.001$ 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-$4^{9\text{-}39}$ with (closed symbols) and without (open symbols) 200umol/kg ghrelin 15 minutes before oral glucose administration and determination of (A) plasma GLP-1, (B) plasma insulin and (C) blood glucose at 0, 10 and 60 minutes (insets: fasting levels; open bar, exendin-$4^{9\text{-}39}$; closed bar, exendin-$4^{9\text{-}39}$ plus ghrelin). Data are presented as mean +/- SE, n=

9. **p<0.01 at individual time points; ##p<0.01 for overall group effect in a Two-factor ANOVA

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

Figure 5. Ghrelin treatment in obese mice restores glucose tolerance and enhances GLP-1 secretion. (A) Fasting ghrelin levels in chow fed (open bar) and HFD (closed bar) mice. (B) Glucose response curve to oral glucose in chow fed (grey symbols), HFD (open symbols) and HFD/ghrelin-treated (closed symbols) mice (inset: fasting levels; grey bar, chow-fed; open bar, HFD-saline; closed bar, HFD-ghrelin); 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 (open symbols) or ghrelin (closed symbols; inset: fasting levels; open bar, saline; closed bar, ghrelin). Data are presented as mean +/- SE, n= 6-9. *p<0.05, **p<0.01, vs chow-fed or at individual time points; #p<0.05 for overall group effect in a Two-factor ANOVA.

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 = 276bp) in whole brain (lane 1) and GLUTag cells (lane 2), and the human ghrelin receptor (predicated size = 172bp) in NCI-H716 cells (lane 3); molecular weight ladder (L). (B-C) Ghrelin treatment stimulates GLP-1 secretion from GLUTag (B) and NCI-H716 (C) cells. (D) Western blot and densitometry of pERK1/2 and total ERK1/2 in GLUTag cells treated with 10nM ghrelin with and without U-0126 for 30 min (a representative blot is shown; n=3). (E) GLP-1 secretion in cells pre-treated with and without U-0126 and then treated with 10nM ghrelin. Data are presented as mean +/- SE, n= 6. *$p<0.05$, **$p<0.01$ vs. control

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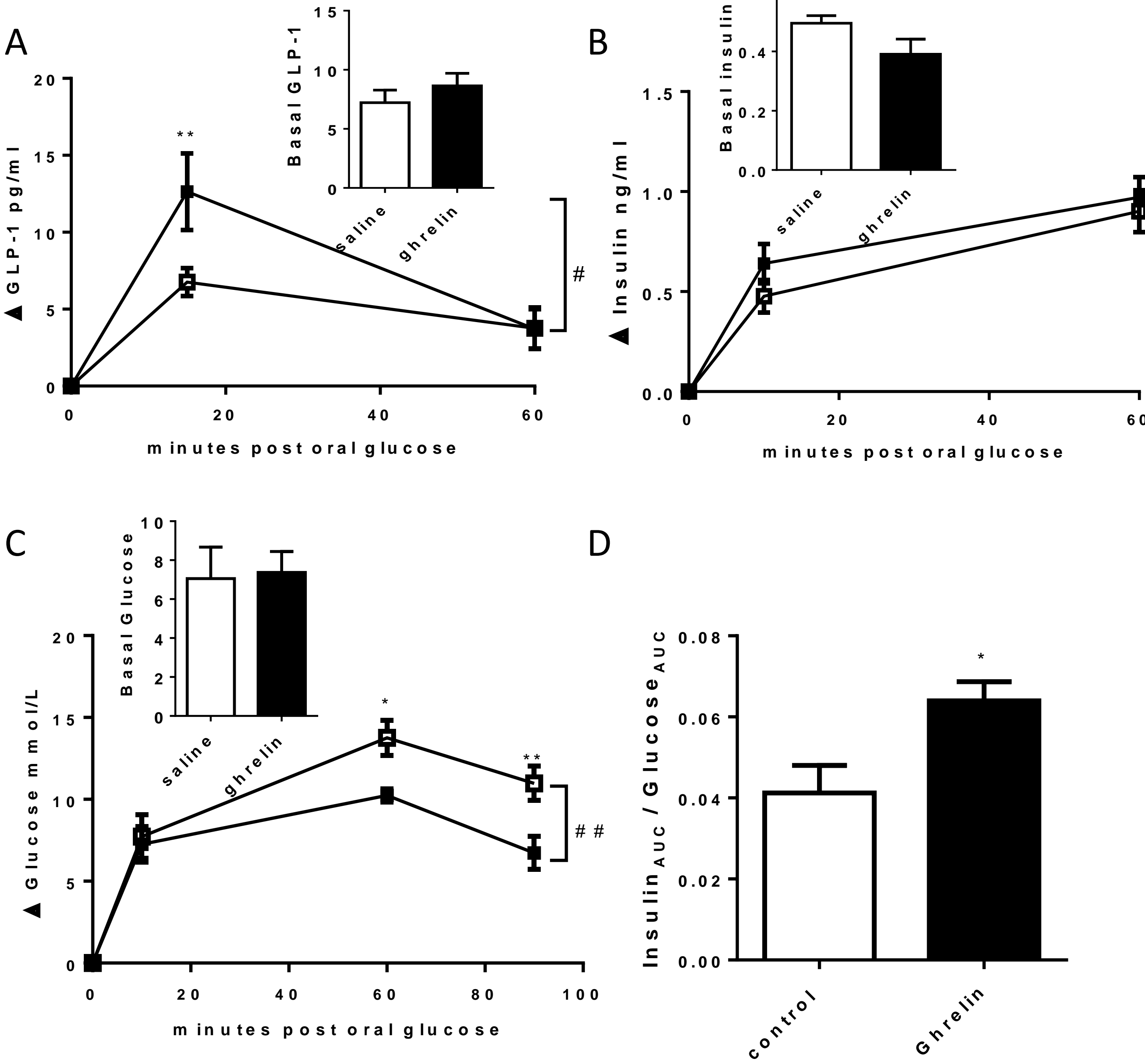
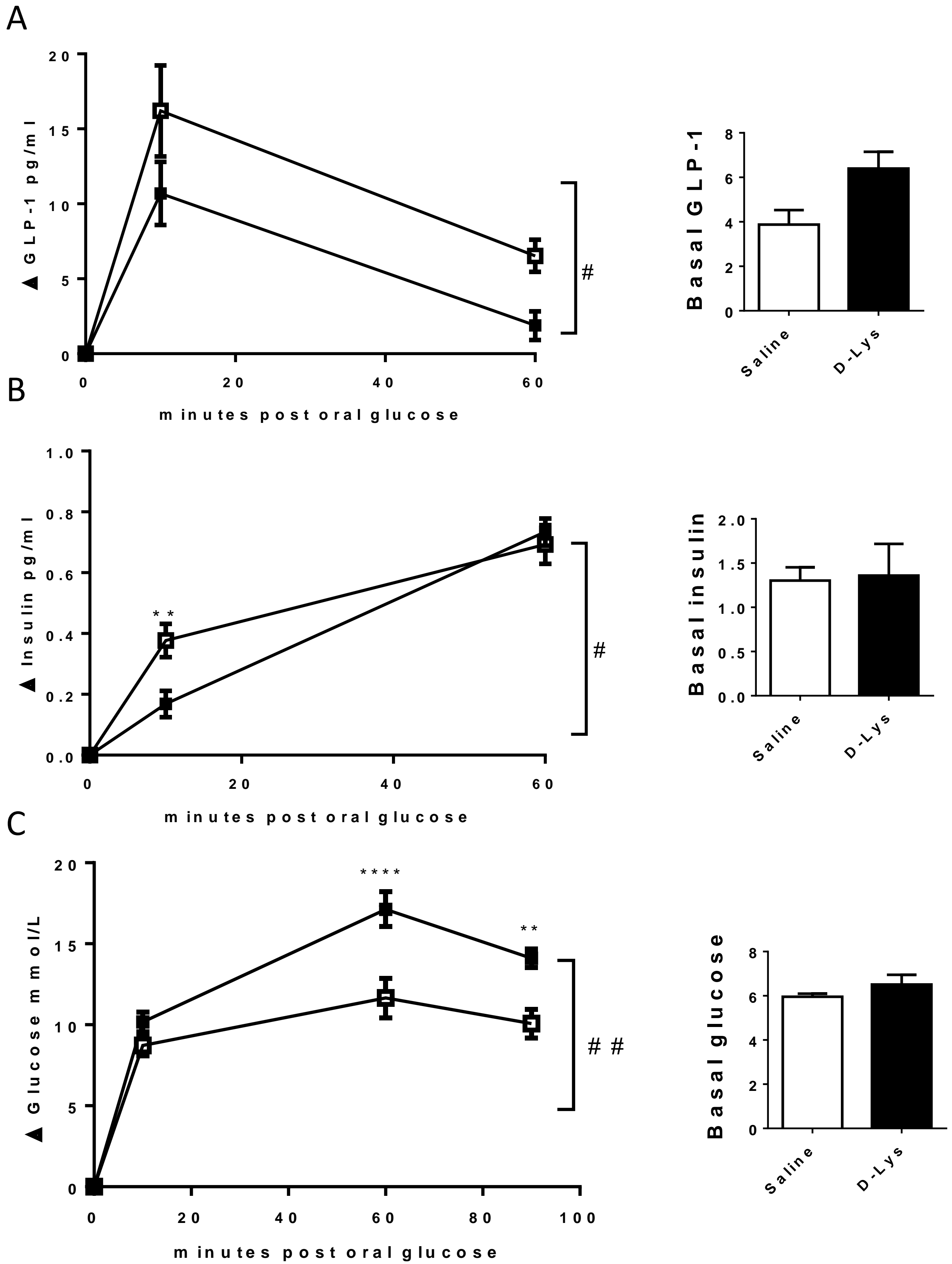


Figure 1



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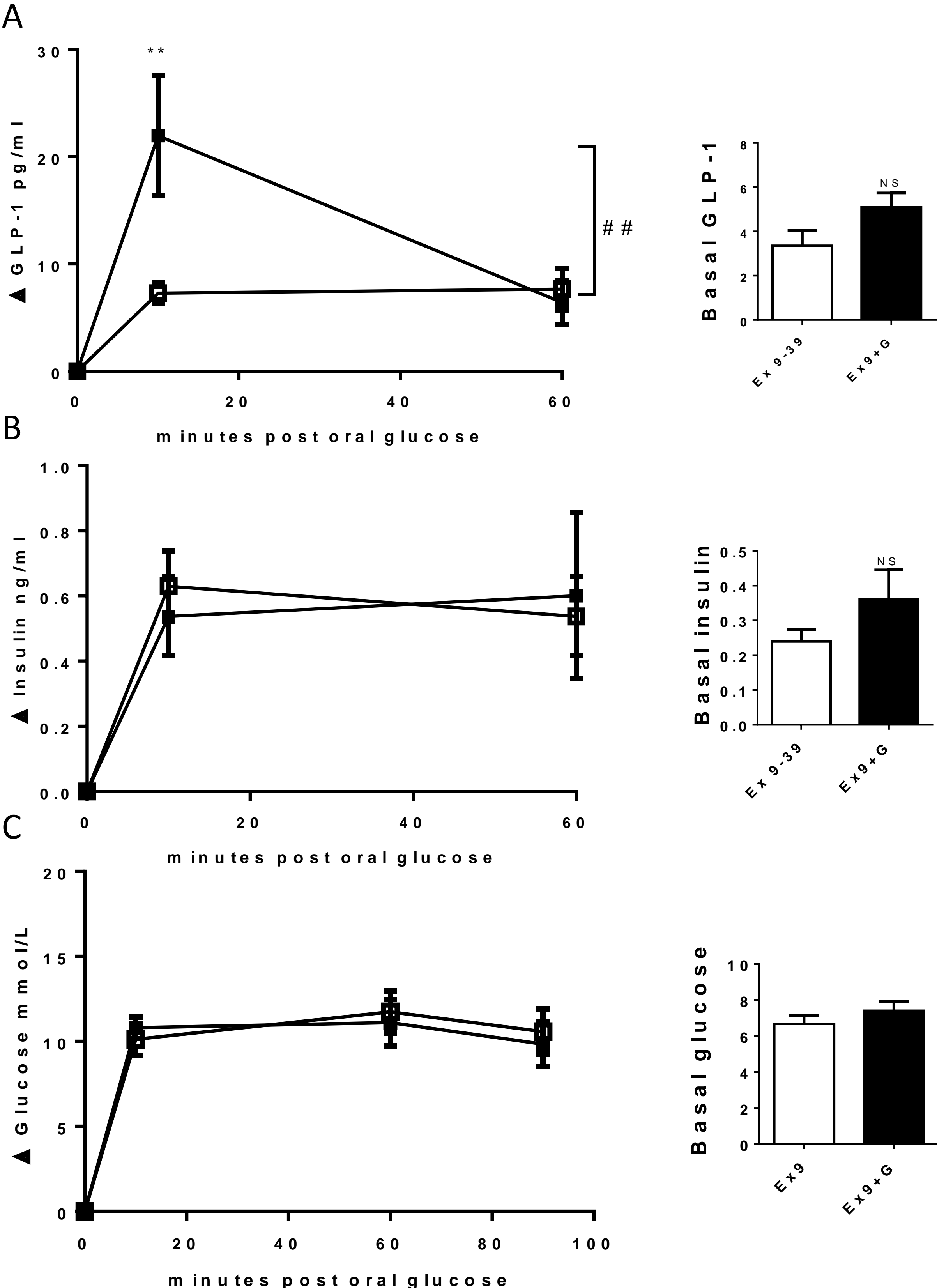

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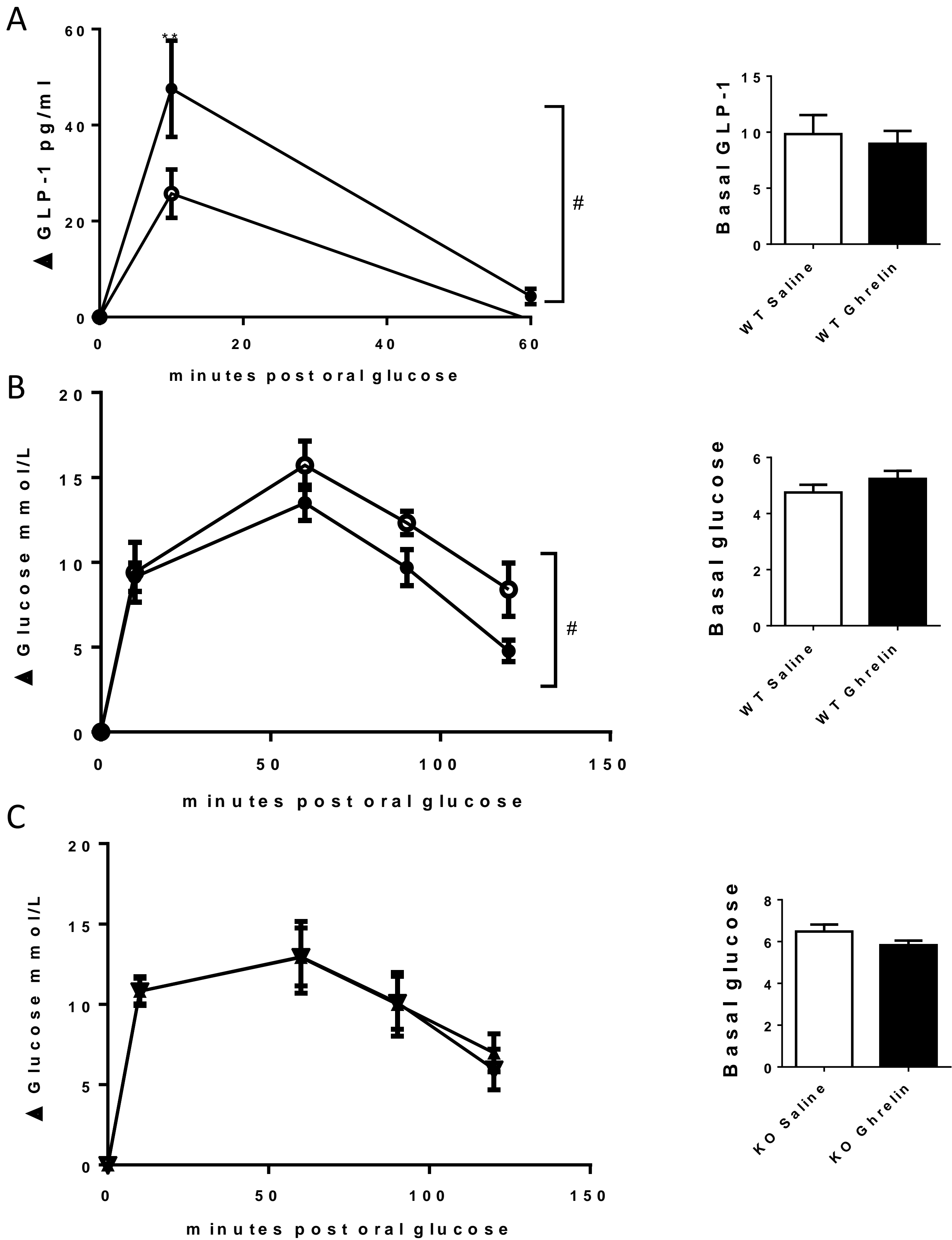

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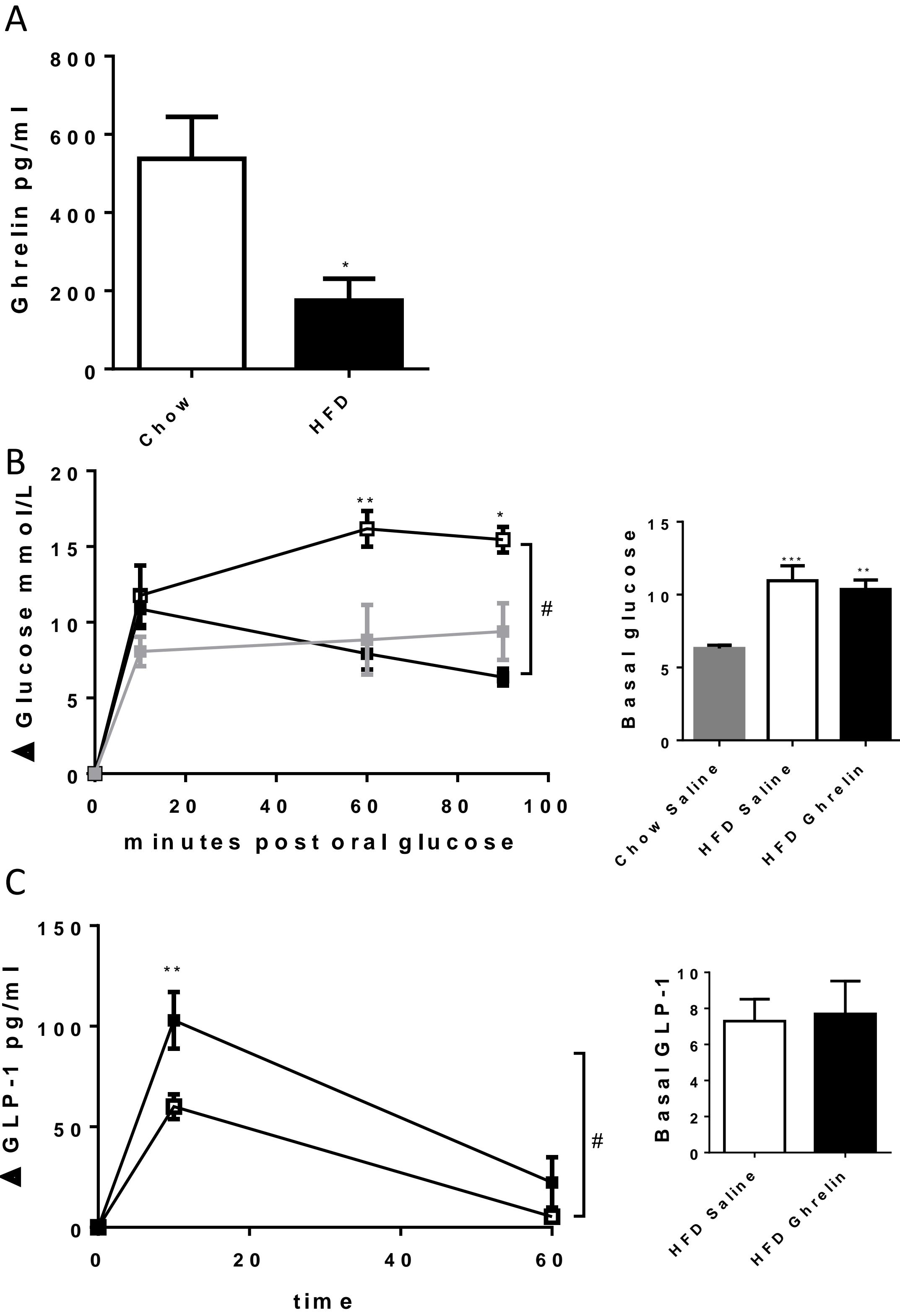

Figure 5

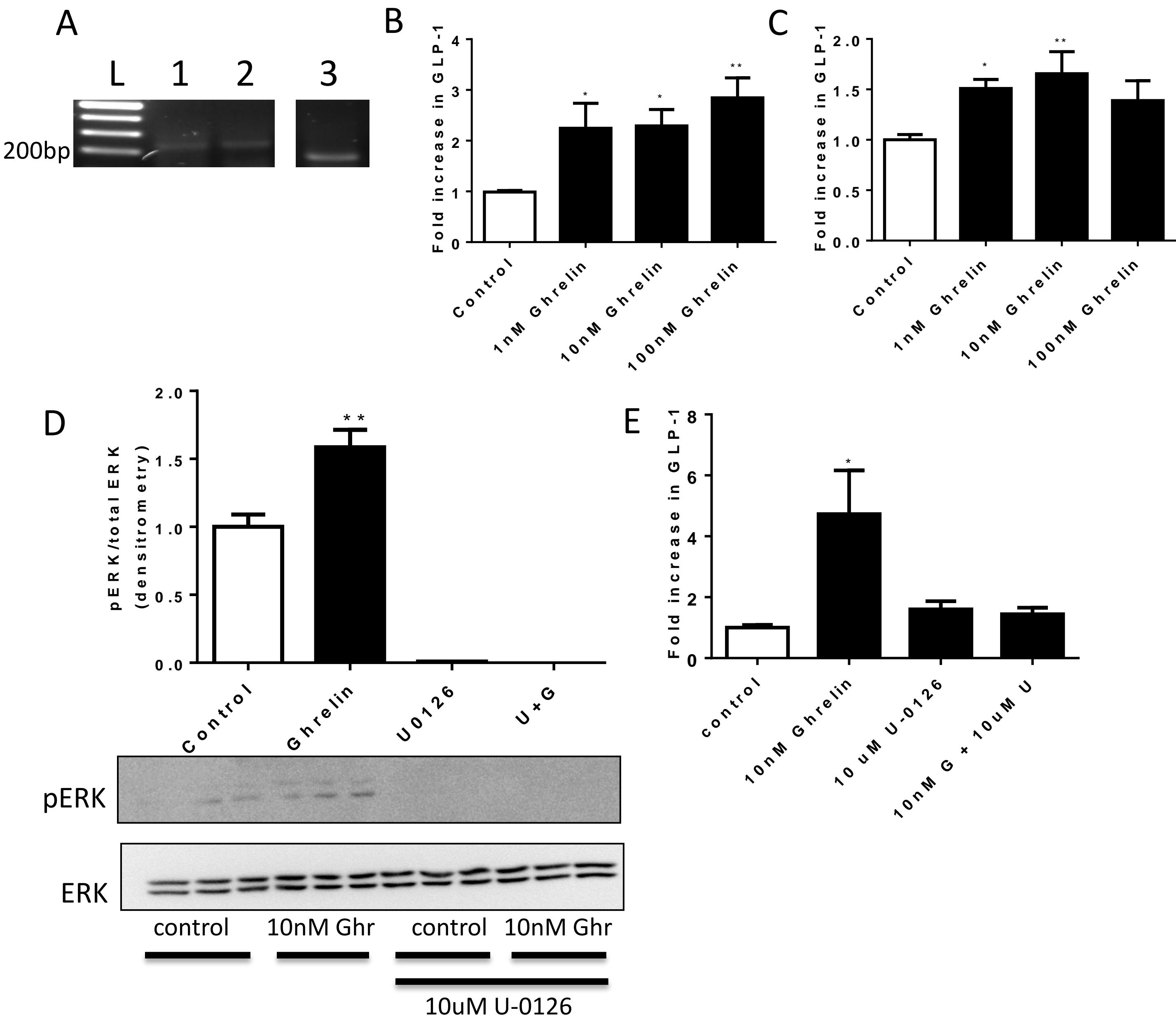

Figure 6