Evidence for paracrine/autocrine regulation of GLP-1-producing cells

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Kappe C, Zhang Q, Holst JJ, Nystrom T, Sjoholm A. Evidence for paracrine/autocrine regulation of GLP-1-producing cells. Am J Physiol Cell Physiol 305: C1041–C1049, 2013. First published August 28, 2013; doi:10.1152/ajpcell.00227.2013.—Glucagon-like peptide-1 (GLP-1), secreted from gut L cells upon nutrient intake, forms the basis for novel drugs against type 2 diabetes (T2D). Secretion of GLP-1 has been suggested to be impaired in T2D and in conditions associated with hyperlipidemia and insulin resistance. Further, recent studies support lipotoxicity of GLP-1-producing cells in vitro. However, little is known about the regulation of L-cell viability/function, the effects of insulin signaling, or the potential effects of stable GLP-1 analogs and dipeptidyl peptidase-4 (DPP-4) inhibitors. We determined effects of insulin as well as possible autocrine action of GLP-1 on viability/apoptosis of GLP-1-secreting cells in the presence/absence of palmitate, while also assessing direct effects on function. The studies were performed using the GLP-1-secreting cell line GLUTag, and palmitate was used to simulate hyperlipidemia. Our results show that palmitate induced production of reactive oxygen species and caspase-3 activity and reduced cell viability are significantly attenuated by preincubation with insulin/exendin-4. The indicated liprotective effect of insulin/exendin-4 was not detectable in the presence of the GLP-1 receptor (GLP-1R) antagonist exendin (9–39) and attenuated in response to pharmacological inhibition of exchange protein activated by cAMP (Epac) signaling, while protein kinase A inhibition had no significant effect. Insulin/exendin-4 also significantly stimulate acute and long-term GLP-1 secretion in the presence of glucose, suggesting novel beneficial effects of insulin signaling and GLP-1R activation on glycemia through enhanced mass of GLP-1-producing cells and enhanced GLP-1 secretion. In addition, the effects of insulin indicate that not only is GLP-1 important for insulin secretion but altered insulin signaling may contribute to an altered GLP-1 secretion.

exendin-4; glucagon-like peptide-1; insulinotrophic; lipotoxicity; L cell

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is an intestinal hormone involved in the regulation of glycemia, which stimulates glucose-dependent insulin secretion (10), and β-cell proliferative, and antiapoptotic pathways, while inhibiting glucagon release, gastric emptying, and food intake (21). The incretin effect is the ability of gastrointestinal hormones such as GLP-1, released in response to food intake, to stimulate insulin release. In healthy individuals, this effect accounts for 50–70% of prandial insulin secretion (21). In type 2 diabetes (T2D), characterized by hyperglycemia resulting from impaired insulin production and insulin resistance in peripheral tissues (20), this incretin response has been suggested to be impaired as a result of reduced postprandial GLP-1/PLA2 gamma concentrations (33). Although some studies have indicated normal GLP-1 secretion in T2D, the conflicting data may be explained by different study populations, where one important difference may be the severity of diabetes (6). In studies examining diabetics with a higher HbA1c, reduced incretin secretion has been shown (30), whereas study populations with lower HbA1c display normal incretin secretion (34). Additionally, when, before initiation of the studies, antihyperglycemic treatment is withdrawn (the length of the washout period), most likely is important for the outcome of the studies (26, 34).

Nonetheless, administration of GLP-1 to T2D patients restores glucose-induced insulin secretion as well as the β-cell sensitivity to glucose and normalizes fasting and postprandial glycemia (21).

GLP-1 is synthesized from the proglucagon gene in enterocyte endocrine L cells. Two major molecular forms of GLP-1 exist: GLP-1 (7–36) amide and GLP-1 (7–37). The majority of circulating biologically active GLP-1 is found in the GLP-1 (7–36) amide form. However, native GLP-1 has a half-life of less than 2 min, due to its degradation by dipeptidyl peptidase-4 (DPP-4) ubiquitously present in plasma, complicating its application as an antidiabetic drug. Stable analogs of GLP-1 such as exendin-4 and liraglutide, as well as DPP-4 inhibitors such as sitagliptin, are available as treatments for T2D. However, enhancing endogenous GLP-1 secretion may be a novel and more physiological option in incretin-based diabetes therapy. Thus it is of great importance to determine the factors that regulate the release of GLP-1 after nutrient ingestion. Many different agents acutely stimulating GLP-1 secretion have been described in the literature, including nutrients, neurotransmitters, and peripheral hormones (31). However, there is still much to be learned when it comes to the regulation of function of the GLP-1-secreting L cells. Since in T2D patients on chronic drug treatment L cells would be exposed to these agents constantly over several years, there is a clear need to investigate the short- and long-term impact of antidiabetic drugs on GLP-1-secreting cells.

Hyperglycemia and hyperlipidemia in T2D are toxic to many cell types, including the insulin-secreting β-cells (18, 35), rendering decreased secretory capacity due to a decreased number of viable cells. We recently reported lipotoxicity also in GLP-1-secreting cells in vitro (14). It is possible that such lipotoxicity contributes to defective GLP-1 secretion in T2D (33) and a GLP-1 secretory response that is progressively diminished with increasing body mass index (23).

Considering the increasing use of stable GLP-1 analogs in T2D therapy, defective insulin signaling in T2D and in many cases eventual insulin replacement therapy, it is important to understand if and how these agents affect intestinal L cells and endogenous GLP-1 secretion in the short and long term. In addition, understanding such effects will also help us understand the molecular mechanisms controlling, and possible paracrine/autocrine regulation of, native intestinal L cells. Such understanding may be a first step towards new and improved diabetes treatments based on increased endogenous GLP-1...
secretion, either through direct effects or through enhanced viability and increased secretory capacity secondary to increased L-cell mass.

In the current study, we sought to determine whether the stable GLP-1 analog exendin-4 and/or insulin exert direct short- and long-term effects on the regulation of GLP-1-secreting cells in terms of function and viability.

MATERIALS AND METHODS

Cell Culture and In Vitro Exposure

The GLP-1-secreting GLUTag cell line (source: glucagon-producing enteroendocrine cell tumor that arose in transgenic mice generated on an out-bred CD-1 background; Ref. 16), graciously donated by Dr. Neil Portwood at Karolinska Institutet, Solna, Sweden, and originally from Dr. Daniel J. Drucker, Mount Sinai Hospital, Samuel Lunenfeld Research Institute, University of Toronto, Toronto, Canada, was cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 5.5 mM glucose, 10,000 U/ml penicillin, and 10 mg/ml streptomycin sulfate (Invitrogen) under 5% CO2. Palmitate (sodium salt; Sigma-Aldrich) exposure media was obtained from Eli Lilly, and exchange protein directly activated by cyclic AMP (Epac) inhibitor ESI-09 was purchased from Biolog Life Science Institute, Bremen, Germany.

MTT Assay

GLUTag cells were plated and cultured in 96-well plates at a density of 136,000 cells/ml for 24 h. Cells were then washed twice with low (2%) serum medium at 5.5 mM glucose. The cells were then treated, in 2% serum medium, with or without insulin or exendin-4 for 48 h in the presence or absence of 0.125 mM palmitate + 0.5% BSA. Viable cell densities were determined by metabolic conversion of the dye MTT (Promega, Madison, WI). Fifteen microliters of the supplied MTT solution were added to each well, and the plates were then incubated for an additional 4 h. The MTT reaction was terminated by dissolving 12.5% ethanol during heating to 60°C. Control cells were given vehicle with equal amounts of ethanol as the palmitate-exposed cells (final concentration of ethanol: 0.03%). Exendin-4 was purchased from Sigma-Aldrich, whereas insulin lispro was obtained from Eli Lilly, and exchange protein directly activated by cyclic AMP (Epac) inhibitor ESI-09 was purchased from Biolog Life Science Institute, Bremen, Germany.

Caspase-3 Activity Assay

GLUTag cells were plated at a density of 250,000 cells/ml and grown in 60-mm Petri dishes for 24 h. Cells were then washed twice with low serum medium (2% FBS and 5.5 mM glucose) before treatment with 0.125 mM palmitate in the presence or absence of insulin or exendin-4 for 48 h. The caspase-3 activity assay kit EnzChek Caspase-3 Assay Kit #1-Z-DEVD-AMC Substrate (Invitrogen) was used according to the manufacturer’s instructions as a measure of apoptosis. Briefly, the caspase-3 colorimetric assay is based on the hydrolysis of a substrate by caspase-3, resulting in the release of fluorescent product, which can be measured at 405 nm. Each experiment was performed in duplicates and repeated at least three times to assess consistency of results.

Hormone Secretion

GLUTag cells were plated at a density of 180,000 cells/ml and grown in 24-well plates for 24–48 h.

Secretion during 48 h. Cells were then treated with exendin-4/insulin at indicated doses for an additional 48 h. Immediately after the 48-h incubation, medium was collected and DPP-4 inhibitor added (10 μM).

Secretion over 2 h. Medium was discarded and the cells were washed with prewarmed glucose free, Krebs-Ringer bicarbonate HEPES (KRBH) buffer/0.2% BSA, followed by a 30-min preincubation with the same buffer. Cells were then treated with/without indicated doses of exendin-4/insulin in the same buffer in the presence or absence of indicated glucose concentrations for 2 h. Immediately thereafter, DPP-4 inhibitor was added and the buffer was collected. Cells were lysed using RIPA buffer, and lysates were analyzed for total protein content (to control for equal number of cells). GLP-1 content in medium/buffer was analyzed using the active GLP-1 (7–36) ELISA Cat. No. EGPL-P35K (Millipore, Billerica, MA). This ELISA is for quantification of biologically active forms of GLP-1 [i.e., GLP-1 (7–36) amide and GLP-1 (7–37)]. The ELISA is based on the capture of active GLP-1 by a monoclonal antibody, which binds specifically to the NH2-terminal region of the active GLP-1 molecule. This ELISA was tested by Millipore for exendin-4 cross-reactivity and no cross-reactivity was seen. In addition, there is very little sequence homology (53%) between exendin-4 and native GLP-1. Only the first nine NH2-terminal amino acids are homologous (barring the second amino acid alanine in GLP-1, which has been replaced with glycine in exendin-4). The GLP-1 determinations were performed according to the manufacturer’s instructions for this ELISA. In addition, for indicated experiments, a GLP-1 total ELISA Cat. No. EZGLPPT-36K (Millipore) was used. This ELISA is for quantification of GLP-1 (7–36 and 9–36) through the use of an anti-GLP-1 polyclonal antibody. All experiments were performed in triplicates and repeated at least three times to assess consistency of results.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR

GLUTag cells were lysed and RNA extracted using Aurum total RNA mini kit (cat. no. 7326820; Bio-Rad Laboratories) according to the manufacturer’s instructions. cDNA was synthesized for qPCR using iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s instructions.

Proglucagon primers were designed according to GATTTTGTGCAGTTGTTGTGAT, ACTTCTTCTCGGAAAGTCCTTCG, using Invitrogen custom primer design software (Invitrogen).

A one-step RT-PCR kit with SYBR Green (iScript one-step RT-PCR kit with SYBR Green; Bio-Rad Laboratories) was used for real-time quantitative RT-PCR. This kit utilizes iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s instructions.

Suppression of GLP-1 Receptor Expression in Cultured Cells Using Short Interfering RNA

Short interfering RNAs (siRNAs) targeting the mouse GLP-1 receptor (GLP-1R), or the corresponding scrambled siRNA (negative control; Santa Cruz Biotechnology, Santa Cruz, CA), were used to suppress GLP-1R expression in GLUTag cells. Cell transfection was performed using AMAXA Nucleofection technology (Lonza, Cologne, Germany). Nucleofection is a technology based on the momentary creation of small pores in cell membranes by applying an electrical pulse. This technique enables nucleic acid substrates deliv

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ERY not only to the cytoplasm but also through the nuclear membrane into the nucleus, allowing for high transfection efficiencies. This technique also makes the transfection success independent from cell proliferation. Fifty to two-hundred picomoles of the siRNA were used for transfection, and Western blotting was used to detect the GLP-1R in cell lysates 48 h after transfection. The rabbit GLP-1R Ab (Abcam, Cambridge, MA) was used to detect the GLP-1R. Experiments were performed in duplicates for each concentration of GLP-1R siRNA to assess consistency of results.

**Analysis of ROS Levels**

Image-iT LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Invitrogen) was used. This kit provides the key reagents necessary for the detection of ROS in live cells. The assay is based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a reliable fluorogenic marker for ROS in live cells. Cellular levels of ROS were measured following exposure to 0.125 mM palmitate in the presence/absence of exendin-4 (13). Each experiment was performed in duplicates and repeated at least three times to assess consistency of results.

**Cell Proliferation Assay**

The effects on cell proliferation were examined by [3H]thymidine incorporation into DNA as a measure of DNA synthesis. GLUTag cells were plated in complete medium (10% FBS, 5.5 mM glucose) and grown in six-well plates at a concentration of 225,000 cells/ml for 24 h. Cells were then washed and preincubated for 20–24 h in low serum medium at low and high glucose (0.5% FBS and 3 mM/11 mM glucose). Following preincubation, the cells were treated with or without exendin-4/insulin for 48 h. Six hours before the end of the incubation, cells were pulsed with 1 μCi/ml [3H]thymidine. Cells were harvested and ultrasonically lysed in milliQ water. DNA was precipitated using TCA, and samples were added to the wells of a beta-counter plate. Scintillation fluid was added and radioactivity determined for all samples. Each experiment was performed in triplicates and repeated at least three times to assess consistency of results.

**Statistical Analysis**

Comparisons between multiple groups were made by a one-way ANOVA. Student-Newman-Keul’s post hoc test was used. Comparisons between control and single treatment groups were done using two-tailed Student’s t-test. P < 0.05 was deemed statistically significant.

**RESULTS**

**GLP-1R Activation on GLP-1-Producing Cells Stimulates Secretion of GLP-1**

To determine putative autocrine effects, as well as whether stable GLP-1 analogs used in T2D therapy can alter viability and/or function of GLP-1-producing cells, we incubated the GLUTag cells with exendin-4. A 2-h incubation with exendin-4 did not significantly modify basal GLP-1 release in the absence of nutrients (data not shown) but dose dependently increased GLP-1 release from the GLUTag cells in the presence of 20 mM glucose (Fig. 1A). This glucose dependency very much resembles the glucose-dependent insulin secretion elicited by GLP-1. A Student’s t-test reveals a significantly increased GLP-1 secretion in response to 10 nM exendin-4 in the presence of 20 mM glucose, compared with 20 mM glucose alone.

In line with direct effects of exendin-4 on these GLP-1-producing cells, we detected 80% decreased GLP-1R immunoactivity in cell lysates transfected with GLP-1R siRNA, compared with untransfected control cells (data not shown). However, as the specificity of the Ab used (Abcam, Cambridge, MA) was recently questioned (9, 24), further studies are necessary to confirm these data.

We also investigated the involvement of the GLP-1R in the exendin-4-stimulated GLP-1 secretion, and, as expected, we found that using the GLP-1R antagonist exendin (9–39) inhibited the exendin-4 stimulation of GLP-1 secretion: a one-way ANOVA indicates loss of a significant effect when exendin-4 is coincubated with exendin (9–39) (Fig. 1B). These data, in conjunction with GLP-1R mRNA expression (data not shown), strongly indicate functional GLP-1R expression on GLUTag cells.

In clinical practice, patients receive stable GLP-1 analogs for long periods of time, and there are reports on GLP-1R desensitization in other cell systems in vitro (32). Consequently, to further evaluate the stimulatory effect of GLP-1R activation by exendin-4 and the glucose dependency of this effect, we went on to determine GLP-1 secretion in response to exendin-4 at four different glucose concentrations. Furthermore, a time-dependent comparison of exendin-4-stimulated GLP-1 secretion was undertaken. For this purpose, cells were exposed to vehicle or exendin-4 in the presence or absence of glucose in medium supplemented with 10% FBS.

In contrast to the stimulatory effect of exendin-4 observed in the presence of KRBH buffer supplemented with 20 mM glucose (Fig. 1A), we could not detect increased amounts of GLP-1 (7–36 and 9–36), irrespective of ambient glucose concentrations, following a 2-h incubation with 10 nM exendin-4 in serum-supplemented medium (data not shown) nor could glucose-stimulated secretion be detected following a 2-h incubation (data not shown). However, the serum-supplemented medium alone, in the absence of glucose, potently and significantly increased the amount of GLP-1 secreted during 2 h (Fig. 1C).

A significant glucose-induced secretion was observed following 24 h and increased in magnitude following 48 h, with a maximum and significant, following ANOVA analysis of data, potentiation of secretion already at the lowest concentration of glucose (Fig. 1, D and E). A stimulatory effect of exendin-4 on GLP-1 was observed following a 48-h incubation in the presence of 5.5 mM glucose (significance was obtained for this effect using Student’s t-test; Fig. 1F), while no effect of exendin-4 could be detected at the other glucose concentrations nor following a 24 h incubation (Fig. 1, F and G).

**Enhanced GLP-1 Secretion Following Long-Term Exposure of GLP-1-Producing Cells to Exendin-4 Stems from Enhanced Proliferation and an Increased Number of GLP-1-Producing Cells**

The detection of a significant exendin-4 effect only after 48 h prompted us to investigate if the effect stemmed from increased proliferation of the cells rendering a greater number of cells capable of secreting GLP-1 after 48 h. Indeed, when the GLP-1 content in the medium was normalized by protein content no significant difference was detected between the groups (Fig. 2A). Further, the enhanced glucose stimulation after 48 h compared with 24 h was lost following protein normalziation (data not shown), indicating involvement of
Fig. 1. Glucagon-like peptide-1 receptor (GLP-1R) activation on GLP-1-producing cells stimulates secretion of GLP-1. A: 2-h incubation with 10 nM exendin-4 in the presence of 20 mM glucose significantly stimulates GLP-1 secretion from GLUTag cells (n = 3, in triplicates). B: the GLP-1R antagonist exendin (9–39) inhibits exendin-4-stimulated GLP-1 secretion from GLUTag cells (n = 3, in triplicates). C: 2-h incubation in DMEM supplemented with 10% FBS significantly enhances GLP-1 secretion from GLUTag cells, independent of glucose. Significant glucose-induced secretion of GLP-1 from GLUTag cells is detected after 24 h (D) and 48 h (E) (n = 3, in triplicates). A significant increase of GLP-1 (7–36 and 9–36) was detected in incubation medium containing 10% FBS and 5.5 mM glucose following a 48-h incubation with 10 nM exendin-4 (F), while no effect of exendin-4 could be detected following a 24 h incubation (G) (n = 3, in triplicates). Bars represent means ± SE. *P < 0.05; **P < 0.01, compared with controls. GLP-1 levels detected for a typical experiment range between 198–283 pM (2.5–3.8 pM/μg protein) after 48 h.
Mechanisms Dependent on GLP-1R Activation and Mediated Lipotoxicity Induced by Simulated Hyperlipidemia and by

proliferative effects in the enhanced response detected after 48 h. Analysis of \(^{3}H\)thymidine incorporation into DNA further indicates glucose-dependent increased proliferation in response to exendin-4 (Fig. 2B). In addition, elevated levels of glucose significantly enhance \(^{3}H\)thymidine incorporation into DNA (Fig. 2C). Statistical significance was obtained for these effects using Student’s \(t\)-test between samples from the control and treatment group.

However, we could not detect a significant increase in the proglucagon mRNA levels in response to exendin-4 following a 48-h incubation (data not shown).

Insulin Significantly Stimulates GLP-1 Secretion

Insulin has previously been shown to acutely stimulate GLP-1 secretion from GLP-1-secreting cells in the presence of glucose (17). Data from the present study are in agreement with these findings, showing an increase in GLP-1 secretion from GLUTag cells following acute exposure to insulin in the presence of glucose (Fig. 3A) but no significant increase in the absence of nutrients (data not shown). In the present study we also determined the long-term effects of insulin on GLP-1 secretion at normoglycemic levels, using a 48-h incubation with 1 nM insulin in the presence of nutrient-supplemented medium and 5.5 mM glucose. We show here that insulin, under these conditions, increased GLP-1 secretion/\(\mu\)g protein also over a 48-h incubation (Fig. 3B). As indicated by Student’s \(t\)-test analysis, the effect of insulin was significant at both time points.

Insulin and Exendin-4 Protect GLP-1-Secreting Cells from Lipotoxicity Induced by Simulated Hyperlipidemia and by Mechanisms Dependent on GLP-1R Activation and Mediated by Exchange Protein Activated by cAMP

As we and others have shown, acute exposure to fatty acids increases GLP-1 secretion (12, 14). However, we recently presented data in support of a lipotoxic effect of simulated hyperlipidemia (long-term exposure to high levels of fatty acids) (14). To evaluate the effects of insulin and exendin-4 on simulated hyperlipidemia in vitro, we determined cell viability and caspase-3 activation in response to insulin/exendin-4 in the presence or absence of simulated hyperlipidemia (0.125 mM palmitate + 0.5% BSA). Both insulin and exendin-4 significantly reduced palmitate-induced caspase-3 activity in GLP-1-secreting cells (Fig. 4A) and counteracted the detrimental effects of long-term exposure to palmitate on the viability of these cells (Fig. 4B). Further, the lipoprotective effect, as determined by caspase-3 activity in response to palmitate in the presence or absence of exendin-4, was lost in the presence of the GLP-1R antagonist exendin (9–39) (Fig. 4C), i.e., ANOVA analysis of data shows loss of a significant reduction of caspase-3 activity in response to exendin-4 following coinubcation with exendin (9–39). Considering the stimulatory effect of insulin on GLP-1 secretion and the lipoprotective effect of exendin-4, we went on to investigate whether the lipoprotective effect of insulin was mediated by enhanced secretion of GLP-1 and increased GLP-1R activation. To this end, we

Insulin significantly stimulates short- and long-term GLP-1 secretion. A: 30-min incubation with 100 pM insulin in the presence of 11 mM glucose significantly stimulates GLP-1 secretion from GLUTag cells (n = 3, in triplicates). B: 48-h incubation with 1–10 nM insulin stimulates GLP-1 secretion from GLUTag cells cultured in complete medium (n = 3, in triplicates). Bars represent means ± SE. \(*P < 0.05\), compared with controls.
determined the effect of insulin on caspase-3 activity following exposure to palmitate for 48 h in the presence or absence of the GLP-1R antagonist exendin (9–39). Data from these studies indicate that the lipoprotective effect of insulin is lost in the presence of exendin (9–39) (Fig. 4D), which would suggest an indirect effect, mediated by the GLP-1R, of insulin on lipopapoptosis. GLP-1/exendin-4 binding to the GLP-1R, a G protein-coupled receptor, induces activation of adenylyl cyclase and elevation of intracellular cAMP levels, which elicits protein kinase A (PKA)-dependent and PKA-independent signal transduction. Therefore, we addressed the role of PKA activation in the lipoprotective effect. Our findings illustrate that pharmacological inhibition of PKA could not significantly alter the reduction in palmitate-induced caspase-3 activity observed in response to exendin-4 (Fig. 4E). Exchange protein activated by cAMP (Epac) is another major transducer of physiological

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Fig. 4. Insulin and exendin-4 protect GLP-1-secreting cells from lipotoxicity induced by simulated hyperlipidemia. A: palmitate increases GLUTag cell caspase-3 activity after a 48-h incubation, while this effect is counteracted by coincubation with 10 nM exendin-4 or 1 nM insulin (n = 4, in duplicates). B: palmitate decreases GLUTag cell viability after a 48-h incubation, while this effect is counteracted by coincubation with 10 nM exendin-4 or 1 nM insulin (n = 3, in triplicates). The reductions of palmitate-induced caspase-3 activity in GLUTag cells in response to 10 nM exendin-4 (C) and 1 nM insulin (D) were lost in the presence of 10 nM exendin (9–39) (n = 3–5, in duplicates). The attenuation of palmitate-induced caspase-3 activity in response to coincubation with 10 nM exendin-4 is independent of PKA (E) while dependent on exchange protein activated by cAMP (Epac) activation (F; n = 3, in duplicates). Bars represent means ± SE. *P < 0.05, ***P < 0.001, compared with controls. #P < 0.05, compared with palmitate-treated cells. NS, no significant difference. Statistical analysis was performed using a one-way ANOVA.
effects of cAMP in mammalian cells and known to be activated by exendin-4 and GLP-1R activation. In contrast to PKA, Epac does not phosphorylate target proteins but acts as a guanine nucleotide exchange factor, and to date two different isoforms, Epac 1 and Epac 2, are known in mammals. ANOVA analyses of our data demonstrate that pharmacological inhibition of both isoforms of Epac significantly attenuates the lipoprotective of exendin-4 (Fig. 4F).

Exendin-4 and Insulin Reduce Palmitate-Induced ROS Production Independent of Exchange Protein Activated by cAMP

We have previously shown that palmitate-induced ROS production mediates the increased caspase-3 activity and lipo-
toxicity observed in response to simulated hyperlipidemia in vitro (13). In addition, exendin-4 has previously been reported to reduce ROS production in other cell systems (29). Consequently, we determined the effect of coincubation with 10 nM exendin-4 and 1 nM insulin on palmitate-induced ROS production in GLP-1-secreting cells. We show here, using one-way ANOVA that both exendin-4 (Fig. 5A) and insulin (Fig. 5B) significantly reduce palmitate-induced ROS production.

As exendin-4 has previously been shown to exert ROS-reducing effects in other cell types through activation of Epac (22), we studied ROS production in response to exendin-4 in the presence/absence of pharmacological inhibition of Epac 1 and 2 under conditions of simulated hyperlipidemia. However, ANOVA evaluation of our data indicate that the ROS-reducing effect observed following a 12-h incubation with exendin-4 under conditions of simulated hyperlipidemia is unaltered in response to pharmacological inhibition of Epac (Fig. 5C).

DISCUSSION

This study provides novel data in support of direct effects, modulating function and viability, of exendin-4 and insulin, suggesting paracrine/autocrine regulation, in GLP-1-secreting cells. Specifically, our data indicate that the GLP-1 mimetic exendin-4 dose dependently increases GLP-1 secretion by direct effects on GLP-1-secreting cells. Similar stimulatory effects are observed in response to insulin, and both agents protect GLP-1-producing cells against lipoapoptosis through mechanisms dependent on GLP-1R activation. The presence of autocrine regulation, i.e., enhanced GLP-1 secretion following activation of the GLP-1R on GLP-1-producing cells, of glucose-induced GLP-1 secretion, as reported in the present study, has previously been indicated (7), and a positive autocrine feedback of GLP-1R activation would be consistent with mechanisms operative in other endocrine cell types, e.g., the insulin-producing β-cells (2). It should be noted that the glucose-dependent stimulation of GLP-1 secretion in response to insulin and exendin-4 indicates that the autocrine/paracrine feed-back loop comes into play only when needed.

The GLUTag cells used throughout this study to determine the effects of insulin and GLP-1R activation on GLP-1-producing cells, are a stable immortalized murine enteroendocrine cell line that expresses the proglucagon gene and secretes the glucagon-like peptides (16). GLUTag cells appear quite well differentiated and recapitulate the responsiveness of primary intestinal cell cultures to physiological and pharmacological GLP-1 secretagogues (3, 11). A drawback of culturing and studying models of the L cells in vitro may be nonuniform distribution of receptors and transporters rendering an apical/basolateral surface well adjusted to the existing microenvironment in native L cells, and the unfeasibility of apical/basolateral exposure to agents in vitro. However, the frequent use of GLUTag cells as a model of the L cell renders these data supporting GLUTag cell GLP-1R expression of importance. Some concentrations of insulin and exendin-4 used herein are supratherapeutical. However, it is important to consider that cells in culture are grown in overabundance of nutrients; 2–10% FBS is added to the media, which results in excessive growth stimulation. This is an inherent limitation of in vitro studies using cell lines and can explain the need for higher concentrations, to detect the effects in cell culture, than what is normally seen in diabetic patients. The concentrations of exendin-4 and insulin are thus in the range of concentrations used previously similar in vitro studies.

We report here that the stimulatory effect on GLP-1 secretion in response to exendin-4 is GLP-1R-dependent, as indicated by loss of the effect in the presence of the GLP-1R antagonist exendin (9–39). Further, the increased GLP-1 se-

Fig. 5. Exendin-4 reduces the reactive oxygen species (ROS) production mediating palmitate-induced lipotoxicity, while the lipoprotective effect is independent of protein kinase A. A: palmitate increases ROS production in GLUTag cells after a 10 h incubation, while this effect is counteracted by coincubation with 10 nM exendin-4 (n = 3, in duplicates) or 1 nM insulin (B) (n = 3, in duplicates). C: reduced ROS production observed in response to 10 nM exendin-4 is independent of Epac, as indicated by a sustained ROS-reducing effect in the presence of 5 μM Epac inhibitor ESI-09 (n = 3, in duplicates). Bars represent means ± SE. *P < 0.05, compared with controls. #P < 0.05, compared with palmitate-treated cells. Statistical analysis was performed using Student’s t-test and a one-way ANOVA.
cretion is observed in absence of increased proglucagon mRNA levels, indicating that the effect involves increased secretion of prestored GLP-1. However, further studies are necessary to determine the exact mechanisms behind the observed effects, and studies using transfection with GLP-1R siRNA should be performed to exclude the possibility of exendin (9–39) exerting effects independent of its inhibitory effect on GLP-1R signaling.

The observed role for insulin in acute glucose-stimulated GLP-1 secretion has previously been shown (17). However, the data from the present study also indicate more long-term effects of insulin signaling on GLP-1 secretion. Future studies will focus on the mechanisms mediating this effect.

T2D patients often have elevated levels of plasma free fatty acids (FFAs; Refs. 15, 28). High levels of FFAs induce insulin resistance and are toxic to many cell types, and we have, using the fatty acid palmitate to simulate hyperlipidemia, previously shown lipotoxicity in GLP-1-secreting cells in vitro (13–14). Palmitate was used to evaluate the effect of high concentrations of FFAs relevant to T2D, as it is the most abundant saturated FFA bound to human serum albumin (27) and is the most studied FFA in other endocrine cell types.

Our findings indicate that the lipoprotective effect in response to insulin is an indirect effect caused by the increased GLP-1 secretion, as the protective effect of insulin, much like the protective effect of exendin-4, is lost in the presence of exendin (9–39). Cross talk between tyrosine kinase receptors (e.g., the insulin receptor) and G protein-coupled receptors (e.g., the GLP-1 receptor) has been implicated in multiple studies (8, 25). This cross talk may occur at different levels and involve receptor function, substrate availability, as well as receptor-activated downstream signaling pathways. In the present study, we propose that insulin, through increased GLP-1 secretion, promotes increased binding of GLP-1 to its receptor, rendering a lipoprotective effect, as also observed in response to exendin-4–induced GLP-1R activation. However, it cannot be excluded that insulin through activation of its receptor and cross talk between the receptors sensitizes GLUTag cells to GLP-1 and amplifies GLP-1R-dependent signaling.

We have previously shown that palmitate induces ROS production and that palmitate-induced lipotoxicity is the result of increased ROS production, where the ROS-sensitive MKK3/6-p38 pathway mediates lipoapoptosis of GLP-1-secreting cells (13). In line with a ROS-induced lipotoxicity and insulin/exendin-4-mediated lipoprotection, we report here that exendin-4 and insulin significantly reduce palmitate-induced ROS production. However, whereas we find the lipoprotective effect of GLP-1R activation in response to exendin-4 to be independent of PKA and mediated by Epac, our studies indicate that the ROS-reducing effect of exendin-4 does not require Epac signaling. This may seem contradictory; nevertheless, it is possible that although exendin-4 reduces ROS through Epac-independent signaling at the time point relevant for this study, the lipoprotective effect ultimately requires Epac-dependent upregulation of pathways mediating oxidative stress resistance. In support for such mechanisms, an Epac-dependent Rit GT-Pase-p38 MAPK signaling pathway has been shown to mediate oxidative stress resistance (5).

Considering the impaired GLP-1 secretion in T2D, contributing to defective postprandial insulin secretion, it would be highly desirable to increase endogenous GLP-1 secretion. Importantly, enhancing endogenous GLP-1 production may be superior to current incretin therapy, as GLP-1 would be released by its native route directly into the portal vein before hepatic passage. This is important, since (like insulin) >80% of GLP-1 is degraded between secretion and posthepatic passage, and there are reports of a GLP-1-regulated glucose sensor in the portal vein that (via nervous signals) controls insulin secretion (4). Further, endogenous GLP-1 secretion has a pulsatile secretory pattern (1) that cannot be mimicked by current incretin therapy. Further, some concern regarding the use of DPP-4 inhibitors in incretin therapy may be valid as DPP-4 is not specific for GLP-1 but also leads to degradation of other hormones, such as GLP-2, insulin-like growth factor-1, substance P, and several cytokines. Such action and/or receptor desensitization could potentially explain why treatment of T2D patients with DPP-4 inhibition reduces GLP-1 secretion, despite positive autocrine feedback in response to GLP-1R activation found here.

Enhancing endogenous GLP-1 secretion could theoretically be done in one of two ways: either acutely by direct effects on hormone secretion or over the long term through protecting/preserving the native GLP-1-secreting cells and thereby obtaining an increased L-cell mass and thus increased GLP-1 secretory capacity. Therefore, the lipoprotective effect of insulin and exendin-4 noted here may be of great importance in enhancing long-term endogenous GLP-1 secretion.

Further studies are necessary to define the role for ROS and the intracellular signaling mediating these protective effects, as these studies may help identify molecular targets for increasing L-cell viability and secretory capacity in T2D. Whether the lipoapoptosis described herein contributes to the GLP-1 deficiency in T2D patients, in whom lipotoxicity often prevails, and whether such an effect could be counteracted by insulin/GLP-1R activation will also need to be further studied in vivo using animal models. In future work, we plan to address this important issue in vivo by treating rodents with exendin-4 and a high-fat diet, followed by immunohistological evaluation of L-cell mass, growth, and apoptosis.

In conclusion, although more data are needed to determine the physiological relevance and molecular mechanism of the direct stimulatory and lipoprotective short- and long-term effects of exendin-4 and insulin on the enteroendocrine GLP-1-secreting cells, this study provides novel and intriguing results suggesting that exendin-4 and insulin by direct effects on GLP-1-secreting cells promote GLP-1 secretion directly and through enhanced viability of GLP-1-secreting cells under conditions of diabetic hyperlipidemia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.K., Q.Z., and A.S. conception and design of research; C.K. and J.J.H. performed experiments; C.K., T.N., and A.S. analyzed data; C.K., Q.Z., T.N., and A.S. interpreted results of experiments; C.K. prepared figures; C.K. drafted manuscript; C.K., J.J.H., T.N., and A.S. edited.
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