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Long-term Exposure of Pancreatic Beta-Cells to Palmitate Results in SREBP-1C-dependent Decreases in GLP-1 Receptor Signaling via CREB and AKT and Insulin Secretory Response.

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Abstract

Objectives. The effects of prolonged exposure of pancreatic beta-cells to high saturated fatty acids on GLP-1 action were investigated.

Methods. Murine islets, human pancreatic 1.1B4 cells and rat INS-1E cells were exposed to palmitate for 24 h. mRNA and protein expression/phosphorylation were measured by real-time RT-PCR and immunoblotting, respectively. Specific siRNAs were used to knockdown expression of the GLP-1 receptor (*Glp1r*) and *Srebf1*. Insulin release was assessed with a specific ELISA.

Results. Exposure of murine islets, as well as of human and INS-1E beta-cells, to palmitate reduced the ability of exendin-4 to augment *insulin* mRNA levels and induce insulin release. In addition, palmitate blocked exendin-4-stimulated CREB and AKT phosphorylation, whereas phosphorylation of MEK-1/2 and ERK-1/2 was not altered. Similarly, RNAi-mediated suppression of *Glp1r* expression prevented exendin-4-induced CREB and AKT phosphorylation, but did not impair exendin-4 stimulation of MEK-1/2 and ERK-1/2. Both islets from mice fed a high fat diet and human and INS-1E beta-cells exposed to palmitate showed reduced GLP-1 receptor and PDX-1 and increased SREBP-1C mRNA and protein levels. Furthermore, suppression of SREBP-1C protein expression prevented the reduction of PDX-1 and GLP-1 receptor levels and restored exendin-4 signaling and action. Finally, treatment of INS-1E cells with metformin for 24 h resulted in inhibition of SREBP-1C expression, increased PDX-1 and GLP-1 receptor levels, consequently, enhancement of exendin-4-induced insulin release.

Conclusion/interpretation. Palmitate impairs exendin-4 effects on beta-cells by reducing PDX-1 and GLP-1R expression and signaling in a SREBP-1C-dependent manner. Metformin counteracts the impairment of GLP-1R signaling induced by palmitate.

Abbreviations

AKT: v-akt murine thymoma viral oncogene homolog

AMPK: AMP-activated protein kinase

CREB: cAMP-response-element-binding protein

FA: fatty acid

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GUSB: glucuronidase beta

JNK: c-Jun N-terminal kinase

MEK: MAP kinase-ERK kinase

PPAR: peroxisome proliferator-activated receptor

RNAi: RNA interfering

RNA18S1: RNA 18s ribosomal 1

RPMI1640: Roswell Park Memorial Institute

siRNA: short interfering RNA

SREBF1: sterol regulatory element binding transcription factor 1

Introduction

GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are major incretins released from gut endocrine cells in response to nutrient ingestion. They have important physiological roles, the most characterized of which is potentiation of glucose-stimulated insulin secretion from the beta-cells, the so-called incretin effect (1). Individuals with type 2 diabetes mellitus typically show an impaired incretin effect (2, 3). However, the secretion of GIP and GLP-1 is not always decreased (4, 5), suggesting that defects in incretin receptor signaling also contribute to this phenomenon. Specifically, the incretin effect was shown to be markedly reduced in type 2 diabetes, primarily because of a defect in beta-cell sensitivity to GIP; on the other hand, the insulinotropic effect of GLP-1 may be preserved although reduced in its magnitude (6). In recent years, clinical experience with GLP-1 analogs, such as exenatide, liraglutide and others, shows that these agents are generally effective in correcting hyperglycemia by stimulating insulin secretion (7) in type 2 diabetes, even though some patients may not show an adequate therapeutic response.

The progressive deterioration in beta-cell function over time in patients with type 2 diabetes, characterized both by beta-cell secretory defects and decreased beta-cell mass, results at least partly from the deleterious effects of high glucose and saturated fatty acid (FA) levels, referred to as beta-cell gluco-lipotoxicity (8). The specific increase of plasma FA is thought to be an important link between obesity and type 2 diabetes (9). Indeed, while acute exposure to elevated plasma FA enhances glucose- and non-glucose stimulated insulin secretion both *in vitro* and *in vivo* (10), long-term exposure to FA impairs glucose-stimulated insulin secretion (GSIS) and may induce beta-cell death (11, 12). The impact of obesity, a condition characterized by increased circulating FA levels, on incretin action is less explored. Because type 2 diabetes is strongly associated with obesity, the question of the separate impact of obesity and hyperglycemia on incretin action has been partially addressed. In recent years, however, it has been shown that obesity and glucose tolerance independently attenuate the incretin effect on beta-cell function, and GLP-1 response specifically (13). Moreover, the incretin effect is significantly reduced in obese compared to lean subjects with

normal glucose tolerance (14), and obesity was recently shown to attenuate the glucose-lowering effect of the dipeptidyl peptidase-4 inhibitor sitagliptin in Japanese patients with type 2 diabetes (15). Finally, elevated saturated FA levels contribute to impaired responsiveness to GLP-1 in rodent insulinoma cell lines and isolated islets (16). Altogether, these results suggests that lipids may be involved in modulating GLP-1 responsiveness in pancreatic beta-cells.

Specific defects at the GLP-1 receptor level may contribute to impaired incretin action in diabetes. Indeed, the expression of the GLP-1 receptor, a member of the G_s-protein-coupled receptor superfamily, is decreased in pancreatic islets cultured in high glucose concentrations for 48 h (17, 18). Whether prolonged exposure to saturated FA also results in reduced GLP-1 receptor expression and which are the mechanisms involved is still unclear. Expression of sterol regulatory element-binding protein (SREBP-1C), a membrane-bound transcription factor of the basic helix loop helix leucine zipper family, established to be a regulator of lipogenic enzymes in the liver (19), is promptly upregulated by dietary intake of saturated FA (20, 21). Moreover, in pancreatic beta-cells, activation of SREBP-1C has been shown to be involved in generation of impaired insulin secretion and glucose intolerance (22, 23). The potential involvement of SREBP-1C in the cross-talk between excess saturated FA and diminished GLP-1 action has not been investigated.

In this study, we show that the chronic exposure of murine islets, as well as of human and rat pancreatic beta-cells, to elevated saturated FA levels is sufficient to impair the effects of exendin-4, a 39-amino acid peptide acting as a full GLP-1 receptor agonist (24), on *insulin* mRNA gene expression and insulin release. Generation of this “incretin resistance” involves reduction of GLP-1 receptor expression via FA-induced activation of SREBP-1C.

Materials and Methods

Cell culture. Rat insulin-secreting INS-1E cells (passage 15-30; a kind gift from C. B. Wollheim, University of Geneva, Geneva, Switzerland) were grown as previously described (25). Human pancreatic insulin-releasing 1.1B4 cells (26) (passage 15-40; purchased from ECACC, European Collection of Cell Cultures, Sigma-Aldrich, St Louis, MO, USA) were grown in monolayer at 37 °C in a humidified incubator gassed with 5% CO_2, in RPMI 1640 medium containing 2 mM L-glutamine supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies, Carlsbad, CA, USA).

Animals. Wild type C57Bl/6 mice were purchased from Charles River Laboratories (Calco, LC, Italy). All animal experimentation respected regulations and guidelines of Italy and European Union and the NIH Principles of Laboratory Animal Care (NIH, publication n. 85-23, revised 1985). All the experiments with mice described in this paper have been evaluated and approved (internal ID 0907) from the Ethics Committee "Comitato Etico per la Sperimentazione Animale" (CESA) of IRSG, Biogem.

Diet and study design. From weaning at the age of 3 weeks onwards, 42 male mice received a standard diet. At the age of 3 weeks, mice were randomly divided as follow: 6 mice were sacrificed at day 0 (0 h); 36 mice received a standard diet or a high-fat diet purchased from Mucedola (Settimio Milanese, Milan, Italy), consisting of 60% energy from hydrogenated palm fat, for an additional 21 days (504 h). Blood samples were collected at the indicated hours from the tail vein of fed mice. Triglyceride concentrations were measured using Triglyceride Quantification Kit (Abcam); the inter-assay coefficient of variation was less than 5%.

Islets isolation and culture. Murine islets were isolated by bile duct perfusion and collagenase digestion as described (27). After isolation, islets were cultured free floating in RPMI 1640 culture medium (Life Technologies, Carlsbad, CA, USA) at 5.5 mmol/l glucose concentration and studied

within 72 h from isolation. Cell viability, measured by Trypan Blue exclusion, was 90% after 72 h in culture.

Treatments. Murine pancreatic islets, human pancreatic 1.1B4 cells and INS-1E cells were pretreated with or without 0.5 mM palmitate (Sigma-Aldrich, St Louis, MO, USA), followed by stimulation with 10 nM or 50 nM exendin-4 (exenatide, from Eli Lilly and Co., Indianapolis, IN, USA). Palmitate was dissolved in 0.1 M NaOH at 70 °C for 30 min, and 5 mM palmitate was complexed with 10% essentially FA-free BSA (FA:BSA molar ratio of 3.3:1). As indicated, cells were pre-incubated with 1,10-phenanthroline (50 μM) for 1 h or with metformin (0.5 mM) for 24 h (both from Sigma-Aldrich, St Louis, MO, USA).

Cell Transfection. INS-1E cells were seeded in 6-well plates. Upon reaching approximately 50% confluence, cells were cultured in growth medium without antibiotics, and after 24 h they were transiently transfected with 30 nM siRNA *Glp1r* (SASI_Rn01_00102001, Rat, NM_012728, Sigma-Aldrich, St Louis, MO, USA) or 100 nM siRNA *Srebf1* (generated within the coding region 5'-GGGGTACTCAAGCCTGCCTTC-3') using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Lipofectamine RNAiMAX only (mock) and negative siRNA (Life Technologies, Carlsbad, CA, USA) controls were carried out in parallel.

Immunoblotting. Cells were lysed in buffer containing 50 mM HEPES (pH 7.4), 1% Triton X 100, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 10 mM NaPP, 10 mM NaF, 4 mM EDTA, supplemented with protease and phosphatase inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets e PhosStop Phosphatase Inhibitor Cocktail Tablets, Roche Diagnostic, Indianapolis, IN, USA). Equal protein samples (40 μg) from the cell lysates were separated on 7% or 10% SDS-PAGE gels, as appropriate, and analyzed by immunoblotting as previously described (25).

Gene expression analyses. RNA isolation was conducted as previously described (28, 29). Genomic DNA contamination was eliminated by DNase digestion (Qiagen, Hilden, Germany), and 500 ng of total RNA was used for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Primers were designed using Primer Express 3.0 (Applied Biosystems, Weiterstadt, Germany): rattus_*Gusb*_For: 5'-GACGTTGGGCTGGTGAACTAC-3'; rattus_*Gusb*_Rev: 5'-CACGGGCCACAATTTTGC-3'; mouse_*Gusb*_For: 5'-CGGAGAGCTCATCTGGAATTTC-3'; mouse_*Gusb*_Rev: 5'-TCCCCTTCTTGTTTCCGATTAC-3'; human_*RNA18S1*_For: 5'-CGAACGTCTGCCCTATCAACTT-3'; human_*RNA18S1*_Rev: 5'-ACCCGTGGTCACCATGGTA-3'; rattus_*insulin*_For: 5'-CTGCCCAGGCTTTTGTCAA-3'; rattus_*insulin*_Rev: 5'-TCCCCACACACCAGGTACAGA-3'; mouse_*insulin*_For: 5'-ACCCACCCAGGCTTTTGTC-3'; mouse_*insulin*_Rev: 5'-TCCCCACACACCAGGTAGAGA-3'; human_*insulin*_For: 5'-TACCAGCATCTGCTCCCTCT-3'; human_*insulin*_Rev: 5'-TGCTGGTTCAAGGGCTTTAT-3'; mouse_*Glp1r*_For: 5'-GGCTCCTCTCCTATCAGGACTCT-3'; mouse_*Glp1r*_Rev: 5'-AGTTGGCTGCCACGCAGTAC- 3'; human_*GLP1R*_For: 5'-ACCTGAACCTGTTTGCATCCTT-3'; human_*GLP1R*_Rev: 5'-GCGGCTGTGCTATACATCCA-3'; mouse_*Pdx1*_For: 5'-CGCGTCCAGCTCCCTTT-3'; mouse_*Pdx1*_Rev: 5'-CCTGCCCACTGGCCGTT-3'; mouse_*Srebf1*_For: 5'-CCACTAGAGGTCGGCATGGT-3'; mouse_*Srebf1*_Rev: 5'-TCCCTTGAGGACCTTTGTCATT-3'. The PCR reactions were carried out in an ABI PRISM 7500 System (Applied Biosystems, Weiterstadt, Germany) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Relative RNA levels were determined by analyzing the changes in SYBR green fluorescence during PCR using the ΔΔCt method. The mRNA level of each gene was normalized using *Gusb* mRNA levels for rat and mouse gene expression analysis, *RNA18S1* mRNA levels for human gene expression analysis.

Assessment of glucose-stimulated insulin secretion. INS-1E cells, human pancreatic cells and isolated mouse islets were preincubated in HEPES-balanced Krebs-Ringer bicarbonate buffer (KRBH) (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgCl_2$, 5 mM KH_2PO_4, 25 mM

211 $NaHCO_3$, 10 mM HEPES and 0.5 % BSA, pH 7.4) containing 3 mM glucose for 1 h, then treated for
212 1 h in KRBH buffer containing the indicated reagents with low (3 mM) or stimulatory (25 mM)
213 glucose concentrations. The supernatants were then obtained and used for subsequent determination
214 of insulin concentrations using an ELISA kit for measurement of rat and mouse insulin (Merck
215 Millipore, Darmstadt, Germany) or human insulin (ALPCO Diagnostic, Salem, NH, USA). The inter-
216 assay and intra-assay coefficients of variation were all <10%; the limits of detection were 0.1 ng/ml
217 for rat and mouse insulin and 5.5 pg/mL for human insulin.
218
219 **Statistical analyses.** All data are presented as means ± SE and were analysed by the Student's t test or
220 ANOVA, as appropriate. Statistical significance was set at *P* value <0.05.
221

Results

Palmitate impairs exendin-4-induced insulin mRNA expression and insulin secretion in pancreatic beta-cells.

To investigate if the incretin effect on beta-cells could be altered by prolonged exposure to high saturated FA concentrations, the biological actions and signaling mechanisms of the GLP-1 analog exendin-4 were investigated in murine pancreatic islets, human pancreatic insulin-releasing 1.1B4 cell lines (HPC) and rat INS-1E cells following pretreatment with 0.5 mM palmitate for 24 h. Stimulation of control murine pancreatic islets with exendin-4 (10 nM or 50 nM) for 1 h resulted in 3-fold increase in *insulin* mRNA levels and 1.7-fold increase in GSIS (P <0.05 vs. cells not treated with exendin-4). By contrast, these responses were impaired in islets exposed to palmitate, showing full inhibition of exendin-4-stimulation of *insulin* mRNA levels and 55% reduction of exendin-4-induced GSIS (P <0.05 vs. cells not treated with palmitate; Fig. 1*A*, *B*). In addition, challenge of murine islets with exendin-4 resulted in increased phosphorylation of the kinases CREB (3-fold), AKT (1.9-fold), and ERK-1/2 MAPK (1.8-fold), and this was evident after 5 min of exposure to the GLP-1 analog (P <0.05 vs. cells not treated with exendin-4; Fig. 1*C*-*E*). However, when murine islets were pretreated with palmitate for 24 h, exendin-4-stimulated phosphorylation of CREB and AKT proteins was abrogated (P <0.05 vs. cells not treated with palmitate; Fig. 1*C*, *D*), whereas phosphorylation of ERK-1/2 kinases was not altered (Fig. 1*E*). Similar results were obtained in human 1.1B4 pancreatic cells and rat INS-1E cells pretreated with 0.5 mM palmitate for 24 h, in which the ability of exendin-4 to augment *insulin* mRNA levels and to enhance GSIS was also inhibited (P <0.05 vs. cells not treated with palmitate; Fig. 1*F*, *G*; Fig. 2*A*, *B*). In addition, pretreatment with palmitate resulted in marked inhibition of CREB and AKT phosphorylation induced by exendin-4 (P <0.05 vs. cells not treated with palmitate; Fig. 1*H*, *I*; Fig. 2*C*, *D*), without altering phosphorylation of MEK-1/2 and ERK-1/2 kinases (Fig. 1*J*; Fig. 2*E*, *F*). The palmitate-dependent impairment of intracellular signaling was specific for the GLP-1 receptor since IGF-I-stimulated AKT phosphorylation was unaltered (Supplemental Fig. 1).

Effects of palmitate on GLP-1 receptor expression in pancreatic beta-cells.

To assess whether palmitate altered GLP-1 signaling and action by interfering with GLP-1 receptor expression, the effects of the saturated FA on GLP-1 receptor protein levels were next investigated. GLP-1 receptor protein levels were significantly reduced by 50% in both murine islets (P <0.05 vs basal; Fig. 3*A*) and human pancreatic beta-cells exposed to palmitate (P <0.05 vs basal; Fig. 3*B*). Similarly, a significant reduction in GLP-1 receptor protein levels was also found in INS1-E cells treated with palmitate up to 48 h (P <0.05 vs basal; Fig. 3*C*). An RNA interference strategy was then used to selectively suppress GLP-1 receptor expression and determine the resulting effects on exendin-4-induced intracellular signaling. INS-1E cells transfected with a siRNA sequence targeting *Glp1r* showed a 45% reduction in GLP-1 receptor protein levels (P <0.05 vs. cells not treated with siRNA*Glp1r*; Fig. 3*D*). Under these conditions, exendin-4-induced phosphorylation of CREB and AKT proteins was fully inhibited (P <0.05 vs. cells not treated with siRNA*Glp1r*; Fig. 3*E*, *F*), but no impairment of exendin-4 stimulation of MEK-1/2 and ERK-1/2 phosphorylation was observed (Fig. 3*G, H*). Altogether, these findings indicate that reduction of GLP-1 receptor levels by approximately 50%, using RNAi technology as well as following prolonged exposure to palmitate, results in impaired exendin-4-induced phosphorylation of CREB and AKT and preservation of exendin-4-induced phosphorylation of MEK-1/2 and ERK-1/2.

Role of SREBP-1C in palmitate-induced impairment of exendin-4 signaling and action.

GLP-1 receptor expression is reported to be regulated by pancreatic duodenal homeobox-1 (PDX-1) (30), and it is also known that SREBP-1C inhibits PDX-1 expression (22, 31). Therefore, we hypothesized that saturated FA may reduce GLP-1 receptor protein levels by inducing SREBP-1C. Indeed, the reduction in *GLP1R* mRNA in response to prolonged palmitate exposure (P <0.05 vs. basal; Fig. 4*A, C*) was associated with a reduction in PDX-1 protein levels by 50% and an increase in SREBP-1C protein levels by 50%, and this was evident in all experimental cells (P <0.05 vs. basal; Fig. 4*B, D, E*). Similar results were observed in islets from mice fed a high-fat diet (HFD). HFD mice showed increased serum triglycerides monitored over a period of 21 days vs. mice fed a standard diet (1.64±0.17 vs. 1.06±0.29 mM; P <0.05), as well as a significant reduction by 35% in *Glp1r* mRNA

levels in their pancreatic islets (P <0.05 vs. islets from control mice; Fig. 5*B*). Moreover, in the pancreatic islets of HFD mice *Pdx1* mRNA levels were decreased by 60% (P <0.05 vs. control mice; Fig. 5*C*) and *Srebf1* mRNA, encoding for SREBP-1C, were increased by 55% (P <0.05 vs. control mice; Fig. 5*D*), in line with the findings in rodent and human beta-cells *in vitro*.

The involvement of SREBP-1C protein in palmitate-induced resistance to exendin-4 was next investigated using both 1,10-phenanthroline, a chemical inhibitor of SREBP-1C activation, and a specific siRNA targeting *Srebf1*. When INS-1E cells were incubated with 1,10-phenanthroline (50 µM for 1 h), the SREBP-1C active protein was markedly reduced (P <0.05 vs. cells not treated with 1,10-phenanthroline; Fig. 6*A*, Supplemental Fig. 2*A*). Under these conditions, palmitate failed to induce SREBP-1C, as well as to reduce PDX-1 and GLP-1 receptor protein levels (P <0.05 vs. cells not treated with 1,10-phenanthroline; Fig. 6*B*, *C*). Consequently, in palmitate-treated beta-cells, 1,10-phenanthroline restored the ability of exendin-4 to increase *insulin* mRNA levels, which was decreased by palmitate (P <0.05 vs. cells treated with palmitate and not with 1,10-phenanthroline; Fig. 6*D*), as well as to stimulate phosphorylation of CREB and AKT proteins (P <0.05 vs. cells treated with palmitate and not with 1,10-phenanthroline; Supplemental Fig. 2*A*, *B*, *C*). Similar results were obtained using an RNAi strategy to selectively suppress *Srebf1*. INS-1E cells transfected with a siRNA specific to *Srebf1* and then treated with palmitate showed a 45% reduction in SREBP-1C protein levels (P <0.05 vs. not treated with siRNA*Srebf1*; Fig. 6*E*). Under these conditions, the inhibition of PDX-1 and GLP-1 receptor protein expression in response to palmitate was no longer observed (P <0.05 vs. cells not treated with siRNA*Srebf1*; Fig. 6*F*, *G*), and the ability of exendin-4 to increase *insulin* mRNA levels was fully preserved (P <0.05 vs. cells not treated with siRNA*Srebf1*; Fig. 6*H*). Altogether, these findings indicate that, in beta-cells exposed to excess saturated FA, the lipid-sensing transcription factor SREBP-1C conveys a signal that, via reductions in PDX-1 and GLP-1 receptor protein expression, alters GLP-1 receptor signaling and GLP-1 receptor agonist action.

Metformin counteracts palmitate-induced resistance to exendin-4 in pancreatic beta-cells.

Palmitate-dependent SREBP-1C activation regulates PDX-1 and GLP-1 receptor expression in pancreatic beta-cells. Since SREBP-1C was shown to be regulated by AMPK in hepatocytes (32), and metformin is known to increase GLP-1 receptor levels in pancreatic beta-cells (33), the effects of metformin on SREBP-1C and GLP-1 receptor in palmitate-treated beta-cell were studied next. Exposure of INS-1E cells to 0.5 mM metformin resulted in a dose-dependent 2-fold increase in AMPK protein phosphorylation, a 30% reduction of SREBP-1C protein expression, and an augmentation of GLP-1 receptor protein levels by 80% (P <0.05 vs. cells not treated with metformin; Fig. 7*A*). Furthermore, in INS-1E cells pretreated with metformin for 24 h, the palmitate-induced increase of SREBP-1C was not observed, and PDX-1 protein content was not reduced but rather augmented (P <0.05 vs. cells not treated with metformin; Fig. 7*B*, *C*). Consequently, in palmitate-treated beta-cells, metformin increased GLP-1 receptor protein levels by 80% (P <0.05 vs. cells not treated with metformin; Fig. 7*D*) and improved the ability of exendin-4 to enhance GSIS (P <0.05 vs. cells not treated with metformin; Fig. 7*E, F*), thus offsetting the saturated FA-dependent impairment of incretin action. These results illustrate the important role of the SREBP-1C/PDX-1 axis in the control of GLP-1 receptor signaling in beta-cells in response to a pharmacological agent used in type 2 diabetes therapy.

Discussion

This study was designed to identify the mechanisms of the reduced incretin effect caused by long-term exposure of pancreatic beta-cells to saturated FA. The degree of saturation of the FA seems to be important for the dysfunction of beta-cells (34), and thus in this study palmitic acid, one of the most abundant saturated FA in human plasma, was used. We show that prolonged exposure of isolated murine pancreatic islets and cultured human and rat pancreatic beta-cell lines to palmitate results in an impaired ability of exendin-4 to promote *insulin* mRNA gene expression and to enhance GSIS, by reducing GLP-1 receptor expression and consequently inhibiting its downstream signaling. In analogy with our finding with excess FA, it was previously demonstrated that hyperglycemia determines a reduction of GLP-1 receptor expression, contributing to impaired exendin-4 effects on beta-cells. The mRNA expression of incretin receptors, *Glp1r* and *Gipr*, were significantly decreased in islets of 90% pancreatectomized hyperglycemic rats, and perfused islets isolated from these animals showed reduced insulin responses to GLP-1 and GIP (17).

Long-term exposure of beta-cells to palmitate inhibited exendin-4-induced CREB and AKT, but not MEK and ERK-1/2 phosphorylation. Changes in activation of specific signaling proteins appear to be the consequence of reduced GLP-1 receptor protein content, which was detected both in human and rat pancreatic beta-cell lines exposed to excess palmitate for up 24-48 h in culture, as well as in pancreatic islets isolated from mice fed with HFD (Figs. 3 and 4). Indeed, *Glp1r* knockdown, obtained with a specific *Glp1r* siRNA and resulting in a reduction of GLP-1 receptor protein content of similar magnitude as that determined by exposure of cells to palmitate, also resulted in inhibition of exendin-4-induced CREB and AKT phosphorylation without affecting MEK and ERK-1/2 activation. These results indicate, for the first time, differential regulation of specific GLP-1 signaling pathways in response to a decrease in the level of GLP-1 receptors in beta-cells. While the CREB/AKT pathway couples the GLP-1 receptor to the insulin secretory pathway, the MEK/ERK-1/2 pathway is preferentially conveying beta-cell growth signals (35) and appears to be activated also in the presence of a more limited number of GLP-1 receptors.

The transcription factor PDX-1 reportedly regulates early pancreatic development and controls the expression of insulin as well as of other beta-cell-specific genes (36, 37). Glucose intolerance due to defective GSIS has been described in the heterozygous *Pdx1*-mutant mouse (38). Interestingly, inhibition of PDX-1 function suppressed the expression of GLP-1 receptor, and this resulted in marked impairment of both basal and exendin-4-stimulated cellular cAMP levels, indicating that PDX-1 may affect insulin secretion by changing cellular cAMP levels and GLP-1 receptor-dependent responses (30). In addition, in INS-1 cells with *Pdx1* knockdown, GLP-1 receptor was down-regulated by approximately 55% at both mRNA and protein levels, further establishing PDX-1 as a determinant of GLP-1 receptor protein expression (39). In line with these previous findings, in this study exposure of murine islets and human and rat beta-cells to palmitate resulted in a reduction of PDX-1 and GLP-1 receptor expression, and this was associated with impairment of exendin-4 signaling and action (Figs. 3 and 4). Additionally, the mechanism through which saturated FA reduced PDX-1 and GLP-1 receptor was shown to involve SREBP-1C, a member of transcriptional factors that regulate genes involved in lipid synthesis (40). Both 1,10-phenanthroline, that prevents SREBP-1C activation by inhibiting the S2P enzyme, and a specific *Srebf1* siRNA could restore exendin-4 effects on *insulin* mRNA expression and insulin release in the presence of palmitate, and this was associated with restored PDX-1 and GLP-1 receptor protein levels. This is consistent with the concept that the palmitate-induced impairment of GLP-1 action in beta-cells involves SREBP-1C-dependent repression of PDX-1 and GLP-1 receptor levels. These results are in agreement with the recent observation by Yang *et al.*, showing SREBP-1C-dependent control of GLP-1 receptor levels in isolated rat islets and INS-1E cells (18). In pancreatic beta-cells, activation of SREBP-1C has been implicated in impaired insulin secretion in response to nutrients, and this is associated with decreased mRNA levels of *Pdx1* and its target genes, including *insulin* (31, 41). Chronic exposure of islets or beta-cells to excess palmitate also lead to blunted GSIS through SREBP-1C (42). Furthermore, Li *et al.* (43) have recently shown that *Srebf1* knockdown prevented the impairment of insulin secretion induced by palmitate in INS-1E cells, and that *Pdx1* mRNA expression was increased in *Srebf1* knockout mice (22). Altogether, these findings suggest that SREBP-1C activation may couple

lipotoxic conditions to impaired insulin secretion and overall beta-cell dysfunction, including the response to GLP-1 receptor agonists, through PDX-1 suppression. Indeed, SREBP-1C and PDX-1 directly interact through basic helix-loop-helix and homeobox domains, respectively, and the SREBP-1C/PDX-1 complex inhibits the recruitment of PDX-1 coactivators (44). The involvement of alternative FA-dependent signals in the impairment of exendin-4 signaling and action has also been proposed. Indeed, saturated FA activate proteins involved in beta-cells damage, including JNK-1/2 (29). However, inhibition of palmitate-induced JNK phosphorylation by SP600125 did not restore the ability of exendin-4 to activate CREB and AKT phosphorylation (Natalicchio et al., data not shown), whereas exendin-4 effects on beta-cell were fully restored when SREBP-1C protein activity was inhibited (Fig. 6, Supplemental Fig. 2).

Preventing SREBP-1C activation is a common event to leptin, metformin and PPAR-γ agonists (32, 45). Thus, there might be a correlation between inhibition of SREBP-1C function and the antidiabetic effects of these agents. Metformin activates AMPK in INS-1E cells and hepatocytes, and activation of AMPK by metformin suppresses hepatic expression of SREBP-1 at both mRNA and protein levels (32, 46). In this study, palmitate-induced resistance to exendin-4 in pancreatic beta-cells could be fully corrected by pretreatment of beta-cells with metformin. Additionally, metformin induced AMPK phosphorylation and lead to a reduction of SREBP-1C protein content in INS-1E cells, thereby preventing the reduction of PDX-1, and actually resulting in increased PDX-1 and GLP-1 receptor protein levels. Previous studies have shown that metformin can restore insulin secretion following chronic exposure of rat islets to non-esterified fatty acids or high glucose through direct regulation of pancreatic beta-cell gene expression (47), including a significant increase in PDX-1 protein levels and its translocation from the cytoplasm to the nucleus (48). However, other investigators have claimed that metformin directly increases GLP-1 receptor expression in INS-1 beta-cells via a PPARα-dependent, AMPK-independent mechanism (49). The reasons for these apparent discrepancies are unknown at present. Interestingly, it was recently demonstrated that PPARα regulates the expression of PDX-1 in INS-1 cells and ameliorates GSIS impaired by palmitate (50). Further studies will have to clarify the potential regulation of SREBP-1C by PPARα. The effects of metformin to correct the

408 impairment of exendin-4 action by palmitate may be mechanistically well suited for combination of
409 metformin with incretin-based therapies, especially in patients with type 2 diabetes and excess
410 circulating saturated FA.
411

412 **References**

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414 1. Shuster LT, Go VL, Rizza RA, O'Brien PC, Service FJ. Incretin effect due to increased
415 secretion and decreased clearance of insulin in normal humans. Diabetes 1988; 37:200–203.
416 2. Nauck M, Stöckmann F, Ebert R, Creutzfeldt W. Reduced incretin effect in type 2 (non-insulin-
417 dependent) diabetes. Diabetologia 1986; 29:46–52.
418 3. Tura A, Muscelli E, Gastaldelli A, Ferrannini E, Mari A. Altered pattern of the incretin effect as
419 assessed by modelling in individuals with glucose tolerance ranging from normal to diabetic.
420 Diabetologia. 2014; 57(6):1199-1203.
421 4. Nauck MA, Vardarli I, Deacon CF, Holst JJ, Meier JJ. Secretion of glucagon-like peptide-1
422 (GLP-1) in type 2 diabetes: what is up, what is down? Diabetologia 2011; 54:10–18.
423 5. Lee S, Yabe D, Nohtomi K, Takada M, Morita R, Seino Y, Hirano T. Intact glucagon-like
424 peptide-1 levels are not decreased in Japanese patients with type 2 diabetes. Endocr J 2010;
425 57:119–126.
426 6. Vilsbøll T, Knop FK, Krarup T, Johansen A, Madsbad S, Larsen S, Hansen T, Pedersen O, Holst
427 JJ. The pathophysiology of diabetes involves a defective amplification of the late-phase insulin
428 response to glucose by glucose-dependent insulinotropic polypeptide-regardless of etiology and
429 phenotype. J Clin Endocrinol Metab 2003; 88:4897–4903.
430 7. Creutzfeldt W. The entero-insular axis in type 2 diabetes--incretins as therapeutic agents. Exp
431 Clin Endocrinol Diabetes 2001; 109 Suppl :S288–303.
432 8. Poitout V, Robertson RP. Minireview: Secondary beta-cell failure in type 2 diabetes--a
433 convergence of glucotoxicity and lipotoxicity. Endocrinology 2002; 143:339–342.
434 9. Paolisso G, Tataranni PA, Foley JE, Bogardus C, Howard B V, Ravussin E. A high
435 concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of
436 NIDDM. Diabetologia 1995; 38:1213–1217.
437 10. Crespin SR, Greenough WB, Steinberg D. Stimulation of insulin secretion by long-chain free
438 fatty acids. A direct pancreatic effect. J Clin Invest 1973; 52:1979–1984.

11. Zhou YP, Grill VE. Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. J Clin Invest 1994; 93:870–876.
12. Natalicchio A, Tortosa F, Labarbuta R, Biondi G, Marrano N, Carchia E, Leonardini A, Cignarelli A, Bugliani M, Marchetti P, Fadini GP, Giorgio M, Avogaro A, Perrini S, Laviola L, Giorgino F. The p66Shc redox adaptor protein is induced by saturated fatty acids and mediates lipotoxicity-induced apoptosis in pancreatic beta cells. Diabetologia 2015; 58:1260–1271.
13. Muscelli E, Mari A, Casolaro A, Camastra S, Seghieri G, Gastaldelli A, Holst JJ, Ferrannini E. Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. Diabetes 2008; 57:1340–1348.
14. Knop FK, Aaboe K, Vilsbøll T, Vølund A, Holst JJ, Krarup T, Madsbad S. Impaired incretin effect and fasting hyperglucagonaemia characterizing type 2 diabetic subjects are early signs of dysmetabolism in obesity. Diabetes Obes Metab 2012; 14:500–510.
15. Bando Y, Kanehara H, Aoki K, Hisada A, Toya D, Tanaka N. Obesity may attenuate the HbA1c-lowering effect of sitagliptin in Japanese type 2 diabetic patients. J Diabetes Investig 2012; 3:170–174.
16. Kang ZF, Deng Y, Zhou Y, Fan RR, Chan JCN, Laybutt DR, Luzuriaga J, Xu G. Pharmacological reduction of NEFA restores the efficacy of incretin-based therapies through GLP-1 receptor signalling in the beta cell in mouse models of diabetes. Diabetologia 2013; 56:423–433.
17. Xu G, Kaneto H, Laybutt DR, Duvivier-Kali VF, Trivedi N, Suzuma K, King GL, Weir GC, Bonner-Weir S. Downregulation of GLP-1 and GIP receptor expression by hyperglycemia: possible contribution to impaired incretin effects in diabetes. Diabetes 2007; 56:1551–1558.
18. Yang Y, Tong Y, Gong M, Lu Y, Wang C, Zhou M, Yang Q, Mao T, Tong N. Activation of PPARβ/δ protects pancreatic β cells from palmitate-induced apoptosis by upregulating the expression of GLP-1 receptor. Cell Signal 2014; 26:268–278.
19. Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Gotoda T, Ishibashi S, Yamada N. Sterol regulatory element-binding

protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. J Biol Chem 1999; 274:35832–35839.

20. Horton JD, Bashmakov Y, Shimomura I, Shimano H. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. Proc Natl Acad Sci U S A 1998; 95:5987–5992.

21. Lin J, Yang R, Tarr PT, Wu P-H, Handschin C, Li S, Yang W, Pei L, Uldry M, Tontonoz P, Newgard CB, Spiegelman BM. Hyperlipidemic effects of dietary saturated fats mediated through PGC-1beta coactivation of SREBP. Cell 2005; 120:261–273.

22. Takahashi A, Motomura K, Kato T, Yoshikawa T, Nakagawa Y, Yahagi N, Sone H, Suzuki H, Toyoshima H, Yamada N, Shimano H. Transgenic mice overexpressing nuclear SREBP-1c in pancreatic beta-cells. Diabetes 2005; 54:492–499.

23. Diraison F, Parton L, Ferré P, Foufelle F, Briscoe CP, Leclerc I, Rutter GA. Over-expression of sterol-regulatory-element-binding protein-1c (SREBP1c) in rat pancreatic islets induces lipogenesis and decreases glucose-stimulated insulin release: modulation by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Biochem J 2004; 378:769–778.

24. Göke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Göke B. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. J Biol Chem 1993; 268:19650–19655.

25. Natalicchio A, De Stefano F, Orlando MR, Melchiorre M, Leonardini A, Cignarelli A, Labarbuta R, Marchetti P, Perrini S, Laviola L, Giorgino F. Exendin-4 prevents c-Jun N-terminal protein kinase activation by tumor necrosis factor-alpha (TNFalpha) and inhibits TNFalpha-induced apoptosis in insulin-secreting cells. Endocrinology 2010; 151:2019–2029.

26. McCluskey JT, Hamid M, Guo-Parke H, McClenaghan NH, Gomis R, Flatt PR. Development and functional characterization of insulin-releasing human pancreatic beta cell lines produced by electrofusion. J Biol Chem 2011; 286:21982–1992.

27. Li D-S, Yuan Y-H, Tu H-J, Liang Q-L, Dai L-J. A protocol for islet isolation from mouse pancreas. Nat Protoc 2009; 4:1649–1652.

494 28. Natalicchio A, Labarbuta R, Tortosa F, Biondi G, Marrano N, Peschechera A, Carchia E,
495 Orlando MR, Leonardini A, Cignarelli A, Marchetti P, Perrini S, Laviola L, Giorgino F.
496 Exendin-4 protects pancreatic beta cells from palmitate-induced apoptosis by interfering with
497 GPR40 and the MKK4/7 stress kinase signalling pathway. Diabetologia 2013; 56:2456–2466.
498 29. Santangelo C, Scipioni A, Marselli L, Marchetti P, Dotta F. Suppressor of cytokine signaling
499 gene expression in human pancreatic islets: modulation by cytokines. Eur J Endocrinol 2005;
500 152:485–489.
501 30. Wang H, Iezzi M, Theander S, Antinozzi P a, Gauthier BR, Halban P a, Wollheim CB.
502 Suppression of Pdx-1 perturbs proinsulin processing, insulin secretion and GLP-1 signalling in
503 INS-1 cells. Diabetologia 2005; 48:720–731.
504 31. Wang H, Maechler P, Antinozzi PA, Herrero L, Hagenfeldt-Johansson KA, Bjorklund A,
505 Wollheim CB. The transcription factor SREBP-1c is instrumental in the development of beta-
506 cell dysfunction. J Biol Chem 2003; 278:16622–16629.
507 32. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii
508 N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in
509 mechanism of metformin action. J Clin Invest 2001; 108:1167–1174.
510 33. Pan QR, Li WH, Wang H, Sun Q, Xiao XH, Brock B, Schmitz O. Glucose, metformin, and
511 AICAR regulate the expression of G protein-coupled receptor members in INS-1 beta cell. Horm
512 Metab Res 2009; 41:799–804.
513 34. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY. Distinct effects of saturated
514 and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 2001; 50:69–76.
515 35. Giorgino F, Laviola L, Leonardini A, Natalicchio A. GLP-1: a new approach for type 2 diabetes
516 therapy. Diabetes Research and Clinical Practice 2006; 74:S152-S155.
517 36. Jonsson J, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas
518 development in mice. Nature 1994; 371:606–609.
519 37. Wang H, Maechler P, Ritz-Laser B, Hagenfeldt KA, Ishihara H, Philippe J, Wollheim CB. Pdx1
520 level defines pancreatic gene expression pattern and cell lineage differentiation. J Biol Chem
521 2001; 276:25279–25286.

522 38. Brissova M, Shiota M, Nicholson WE, Gannon M, Knobel SM, Piston DW, Wright CVE,
523 Powers AC. Reduction in pancreatic transcription factor PDX-1 impairs glucose-stimulated
524 insulin secretion. J Biol Chem 2002; 277:11225–11232.
525 39. Shao S, Liu Z, Yang Y, Zhang M, Yu X. SREBP-1c, Pdx-1, and GLP-1R involved in palmitate-
526 EPA regulated glucose-stimulated insulin secretion in INS-1 cells. J Cell Biochem 2010;
527 111:634–642.
528 40. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by
529 proteolysis of a membrane-bound transcription factor. Cell 1997; 89:331–340.
530 41. Shimano H, Amemiya-Kudo M, Takahashi A, Kato T, Ishikawa M, Yamada N. Sterol
531 regulatory element-binding protein-1c and pancreatic beta-cell dysfunction. Diabetes Obes
532 Metab 2007; 9 Suppl 2:133–139.
533 42. Kato T, Shimano H, Yamamoto T, Ishikawa M, Kumadaki S, Matsuzaka T, Nakagawa Y,
534 Yahagi N, Nakakuki M, Hasty AH, Takeuchi Y, Kobayashi K, Takahashi A, Yatoh S, Suzuki H,
535 Sone H, Yamada N. Palmitate impairs and eicosapentaenoate restores insulin secretion through
536 regulation of SREBP-1c in pancreatic islets. Diabetes 2008; 57:2382–2392.
537 43. Li J, Liu X, Ran X, Chen J, Li X, Wu W, Huang H, Long Y, Liang J, Cheng J, Tian H. Sterol
538 regulatory element-binding protein-1c knockdown protected INS-1E cells from lipotoxicity.
539 Diabetes Obes Metab 2010; 12:35–46.
540 44. Amemiya-Kudo M, Oka J, Takeuchi Y, Okazaki H, Yamamoto T, Yahagi N, Matsuzaka K,
541 Okazaki S, Osuga J, Yamada N, Murase T, Shimano H. Suppression of the pancreatic duodenal
542 homeodomain transcription factor-1 (Pdx-1) promoter by sterol regulatory element-binding
543 protein-1c (SREBP-1c). J Biol Chem 2011; 286:27902–27914.
544 45. Kakuma T, Lee Y, Higa M, Wang Z w, Pan W, Shimomura I, Unger RH. Leptin, troglitazone,
545 and the expression of sterol regulatory element binding proteins in liver and pancreatic islets.
546 Proc Natl Acad Sci U S A 2000; 97:8536–8541.
547 46. Langelueddecke C, Jakab M, Ketterl N, Lehner L, Hufnagl C, Schmidt S, Geibel JP, Fuerst J,
548 Ritter M. Effect of the AMP-kinase modulators AICAR, metformin and compound C on insulin

549 secretion of INS-1E rat insulinoma cells under standard cell culture conditions. Cell Physiol
550 Biochem 2012; 29:75–86.
551 47. Patanè G, Piro S, Rabuazzo AM, Anello M, Vigneri R, Purrello F. Metformin restores insulin
552 secretion altered by chronic exposure to free fatty acids or high glucose: a direct metformin
553 effect on pancreatic beta-cells. Diabetes 2000; 49:735–740.
554 48. Richardson H, Campbell SC, Smith SA, Macfarlane WM. Effects of rosiglitazone and
555 metformin on pancreatic beta cell gene expression. Diabetologia 2006; 49:685–696.
556 49. Maida A, Lamont BJ, Cao X, Drucker DJ. Metformin regulates the incretin receptor axis via a
557 pathway dependent on peroxisome proliferator-activated receptor-α in mice. Diabetologia 2011;
558 54:339–349.
559 50. Sun Y, Zhang L, Gu HF, Han W, Ren M, Wang F, Gong B, Wang L, Guo H, Xin W, Zhao J,
560 Gao L. Peroxisome proliferator-activated receptor-alpha regulates the expression of
561 pancreatic/duodenal homeobox-1 in rat insulinoma (INS-1) cells and ameliorates glucose-
562 induced insulin secretion impaired by palmitate. Endocrinology 2008; 149:662–671.
563

Figure Legends

Figure 1. Effects of palmitate on the biological effects and signaling mechanisms of exendin-4 in murine islets and human cells. Murine pancreatic islets and human pancreatic 1.1B4 cells were incubated with or without 0.5 mM palmitate for 24 h, and then exposed to 10 nM or 50 nM exendin-4 for 1 h (***A***, ***B***, ***F***, ***G***), for 15 min (***C***-***E***), or for indicated times (***H***-***J***). ***A***, ***B***, ***F***, ***G***: Effects of palmitate on exendin-4-induced *insulin* mRNA expression and insulin secretion, respectively, in murine pancreatic islets and human pancreatic beta-cells. ***A***, ***F***: *Insulin* mRNA levels from murine islets (***A***) and human cells (***F***) were evaluated by quantitative real-time RT-PCR and normalized using *Gusb* or *RNA18S1* mRNA, respectively, as internal control (n = 4 experiments). ***B***, ***G***: Murine islets ***(B)*** or human pancreatic 1.1B4 cells ***(G)*** were pretreated with 0.5 mM palmitate for 24 h or left untreated, and then incubated in KRBH buffer containing 3 or 25 mM glucose in the presence or absence of 50 nM exendin-4 for 1 h. Insulin secretion was evaluated by measuring insulin concentrations in the conditioned medium with an ELISA assay (n = 4 experiments). ***C***-***E***, ***H***-***J***: Effects of palmitate on phosphorylation of CREB, AKT and ERK-1/2 MAPK in murine pancreatic islets and human pancreatic beta-cells. Representative immunoblots of CREB phosphorylation and GAPDH protein content and relative ratio of phosphorylated CREB to GAPDH in murine islets (***C***) and human beta-cells (***H***), AKT phosphorylation and AKT protein content and relative ratio of phosphorylated AKT to total AKT in murine islets (***D***) and human cells (***I***), ERK-1/2 phosphorylation and ERK-1/2 protein content and relative ratio of phosphorylated ERK-1/2 to total ERK-1/2 in murine islets (***E***) and human cells (***J***). At least n = 4 independent experiments for each endpoint were performed. *P <0.05 vs. islets or cells not exposed to exendin-4; #P <0.05 vs. islets or cells not treated with palmitate; †P <0.05 vs. islets or cells incubated in 3 mM. Palm, palmitate; Ex-4, exendin-4.

Figure 2. Effects of palmitate the biological effects and signaling mechanisms of exendin-4 in rat insulin-secreting cells. INS-1E cells were incubated with or without 0.5 mM palmitate for 24 h and then exposed to 10 nM or 50 nM exendin-4 for 1 h (***A***, ***B***), or for indicated times (***C***-***F***). ***A***, ***B***: Effects of palmitate on exendin-4-induced *insulin* mRNA expression and insulin secretion. ***A***: *Insulin* mRNA

levels from were evaluated by quantitative real-time RT-PCR and normalized using *Gusb* mRNA as internal control (n = 5 experiments). ***B***: INS-1E cells were pretreated with 0.5 mM palmitate for 24 h or left untreated, and then incubated in KRBH buffer containing 3 or 25 mM glucose in the presence or absence of 50 nM exendin-4 for 1 h. Insulin secretion was evaluated by measuring insulin concentrations in the conditioned medium with an ELISA assay (n = 4 experiments). ***C-F***: Effects of palmitate on phosphorylation of CREB, AKT and ERK-1/2 MAPK. Representative immunoblots of CREB phosphorylation and GAPDH protein content and relative ratio of phosphorylated CREB to total GAPDH (***C***), AKT phosphorylation and AKT protein content and relative ratio of phosphorylated AKT to total AKT (***D***), MEK-1/2 phosphorylation and MEK-1/2 protein content and relative ratio of phosphorylated MEK-1/2 to total MEK-1/2 (***E***), ERK-1/2 phosphorylation and ERK-1/2 protein content and relative ratio of phosphorylated ERK-1/2 to total ERK-1/2 (***F***) in INS-1E cells. At least n = 5 independent experiments for each endpoint were performed. *P <0.05 vs. cells not exposed to exendin-4; #P <0.05 vs. cells not treated with palmitate; †P <0.05 vs. cells incubated in 3 mM glucose. Palm, palmitate; Ex-4, exendin-4.

Figure 3. ***A-C***: Effects of palmitate on GLP-1 receptor protein in murine pancreatic islets, human beta-cells and rat pancreatic beta-cells. Representative immunoblots of GLP-1 receptor protein content in murine islets (***A***), human pancreatic 1.1B4 cells (***B***) and rat INS-1E insulin-secreting cells (***C***). GAPDH was measured to assess protein loading. The ratio of GLP-1 receptor to GAPDH is also shown. At least n = 4 independent experiments were performed. #P <0.05 vs. cells not treated with palmitate. Palm, palmitate. ***D-H***: Role of GLP-1 receptor in exendin-4 signaling in rat insulin-secreting cells. INS-1E cells were transfected with a siRNA sequence targeting *Glp1r* (si*Glp1r*, 30 nM), with a negative control siRNA (siCTRLneg, 30 nM), or with transfection reagents only (Mock), as described under Research Design and Methods. ***D***: Representative immunoblots showing GLP-1 receptor protein expression in INS-1E cells. GAPDH was analyzed as control for equal protein loading (n = 4 experiments). ***E-H***: Effects of siRNA-mediated knockdown of *Glp1r* on exendin-4 signaling. Representative immunoblots and quantitation of multiple experiments showing CREB phosphorylation and relative ratio of phosphorylated CREB to GAPDH (***E***), AKT phosphorylation

and protein content and relative ratio of phosphorylated AKT to total AKT (***F***), MEK-1/2 phosphorylation and protein content and relative ratio of phosphorylated MEK-1/2 to total MEK-1/2 (***G***), ERK-1/2 phosphorylation and protein content and relative ratio of phosphorylated ERK-1/2 to total ERK-1/2 (***H***) in INS-1E cells (n = 5 experiments). *P <0.05 vs. cells not exposed to exendin-4; §P <0.05 vs. cells not treated with si*Glp1r*. Ex-4, exendin-4; GLP-1R, GLP-1 receptor.

Figure 4. Effects of palmitate on GLP-1 receptor (*Glp1r*) mRNA and PDX-1 and SREBP-1C protein expression in murine pancreatic islets and human and rat pancreatic beta-cells. ***A***, ***C***: Effects of palmitate on *Glp1r* mRNA levels in human pancreatic 1.1B4 (***A***) and rat insulin-secreting INS1-E beta-cells (***C***), evaluated by quantitative real-time RT-PCR and normalized using *RNA18S1* and *Gusb* mRNA, respectively, as internal control. ***B***, ***D***, ***E***: Effects of palmitate on PDX-1 and SREBP-1C proteins in human pancreatic 1.1B4 (***B***), rat insulin-secreting INS-1E cells (***D***), and murine pancreatic islets (***E***). Representative immunoblots of PDX-1 and SREBP-1C protein content and quantitation of multiple experiments are shown. GAPDH was measured to assess protein loading and to assess the ratio of PDX-1 and SREBP-1C to GAPDH. At least n = 5 independent experiments were performed. Experimental cells and islets were exposed to 0.5 mM palmitate for indicated times. #P < 0.05 vs. cells not treated with palmitate. Palm, palmitate.

Figure 5. Effects of high-fat diet (HFD) on GLP-1 receptor (*Glp1r*), *Pdx1* and *Srebf1* mRNA levels in mouse pancreatic islets. ***A***: Triglycerides in serum of mice fed with high-fat diet (HFD, black circles) or standard diet (SD, white circles) for different hours (n = 3 each). *Glp1r* (***B***), *Pdx1* (***C***) and *Srebf1* (***D***) mRNA levels in islets from mice fed with high-fat diet (HFD, black circles) or standard diet (SD, white circles) for different hours (n = 3 each). mRNA expression was evaluated by quantitative real-time RT-PCR and normalized using *Gusb* mRNA as internal control. *P <0.05 vs. mice fed a SD.

Figure 6. Role of SREBP-1C in palmitate-induced alterations of exendin-4 action in rat pancreatic beta-cells. ***A-C***: Effects of 1,10-phenanthroline on SREBP-1C, PDX-1 and GLP-1 receptor in rat INS-1E insulin-secreting cells. INS-1E cells were pre-treated with 1,10-phenanthroline (50 µM for 1 h)

and then exposed to 0.5 mM palmitate for 24 h. Representative immunoblots of active SREBP-1C (***A***), PDX-1 (***B***) and GLP-1 receptor (***C***) protein content in INS-1E cells. GAPDH was measured to assess protein loading. The ratio of GLP-1R, PDX-1 and SREBP-1C to GAPDH is also shown. At least $n = 5$ independent experiments were performed for each protein of interest. #P <0.05 vs. control cells not exposed to palmitate; §P 0.05 vs. cells not treated with 1,10-phenanthroline. ***D***: Effects of 1,10-phenanthroline on *insulin* mRNA levels. *Insulin* mRNA levels were evaluated by quantitative real-time RT-PCR and normalized using *Gusb* mRNA as internal control ($n = 5$ experiments). *P <0.05 vs. cells not exposed to exendin-4; #P <0.05 vs. cells not treated with palmitate; §P <0.05 vs. cells not treated with 1,10-phenanthroline. ***E-G***. Effects of siRNA-mediated knockdown of *Srebf1* on SREBP-1C, PDX-1 and GLP-1 receptor in rat INS-1E insulin-secreting cells. INS-1E cells were transfected with a siRNA sequence targeting *Srebf1* (si*Srebf1*, 100 nM), with a negative control siRNA (siCTRLneg, 30 nM), or with transfection reagents only (Mock), as described under Research Design and Methods, and then exposed to 0.5 mM palmitate for 24 h. Representative immunoblots and the quantitation of multiple experiments are shown. GAPDH was analyzed as control for equal protein loading. Representative immunoblots and quantitation of multiple experiments of SREBP-1C (***E***), PDX-1 (***F***) and GLP-1 receptor (***G***) protein content. GAPDH was measured to assess protein loading. The ratio of SREBP-1C, PDX-1 and GLP-1 receptor to GAPDH is also shown. At least $n = 4$ independent experiments for each protein of interest were performed. #P <0.05 vs. cells not exposed to palmitate; §P <0.05 vs. cells not treated with si*Srebf1*. ***H***: Effects of si*Srebf1* on *insulin* mRNA levels. INS-1E cells were pre-treated with si*Srebf1*, exposed to 0.5 mM palmitate for 24 h, and then stimulated with 10 nM exendin-4 for 1 h. *Insulin* mRNA levels were evaluated by quantitative real-time RT-PCR and normalized using *Gusb* mRNA as internal control ($n = 4$ experiments). *P <0.05 vs. cells not exposed to exendin-4; #P <0.05 vs. cells not treated with palmitate; §P <0.05 vs. cells not treated with si*Srebf1*. Palm, palmitate; Ex-4, exendin-4; GLP-1R, GLP-1 receptor; Phe, 1,10-phenanthroline.

Figure 7. Effects of metformin on palmitate-induced alterations of exendin-4 action in rat insulin-secreting INS-1E cells. ***A***: Effects of metformin on AMPK phosphorylation and SREBP-1C and GLP-

1 receptor proteins. Cells were incubated with metformin for the indicated doses for 24 h or left untreated. Representative immunoblots of AMPK phosphorylation, SREBP-1C and GLP-1 receptor protein content. GAPDH was measured to assess protein loading. The ratio of AMPK, SREBP-1C and GLP-1R to GAPDH is also shown. At least $n = 5$ independent experiments were performed. ‡ $P < 0.05$ vs. basal. ***B-D***: Effects of metformin on SREBP-1C, PDX-1 and GLP-1 receptor proteins in INS-1E cells exposed to palmitate. Cells were incubated with 0.5 mM metformin for 24 h and then exposed to 0.5 mM palmitate for 24 h. Representative immunoblots of SREBP-1C (***B***), PDX-1(***C***) and GLP-1 receptor (***D***) protein content. GAPDH was measured to assess protein loading. The ratio of SREBP-1C, PDX-1 and GLP-1 receptor to GAPDH is also shown ($n = 5$). #$P < 0.05$ vs. cells not exposed to palmitate; §$P < 0.05$ vs. cells not treated with metformin. ***E, F***: Effects of metformin on insulin secretion in INS-1E cells exposed to palmitate. Cells were incubated with 0.5 mM metformin for 24 h and then exposed to 0.5 mM palmitate for 24 h. Then, they were incubated in KRBH buffer containing 3 or 25 mM glucose in the presence or absence of 50 nM exendin-4 for 1 h. Insulin secretion was evaluated by measuring insulin concentrations in the conditioned medium with an ELISA assay ($n = 4$). Data are expressed as insulin secreted on total protein content (***E***) or fold change secretion normalized to 3 mM glucose (GSIS) (***F***). †$P < 0.05$ vs. cells incubated in 3 mM glucose; *$P < 0.05$ vs. cells not treated with exendin-4; #$P < 0.05$ vs. cells not treated with palmitate; §$P < 0.05$ vs. cells not treated with metformin. Palm, palmitate; Ex-4, exendin-4; GLP-1R, GLP-1 receptor; Metf, metformin.