Glucolipotoxicity and GLP-1 secretion

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ABSTRACT

Introduction The concept of glucolipotoxicity refers to the combined, deleterious effects of elevated glucose and/or fatty acid levels.

Research design and methods To investigate the effects of chronic glucolipotoxicity on glucagon-like peptide-1-(7-36) amide (GLP-1) secretion, we generated glucolipotoxic conditions in human NCI-H716 enterocrinous cells using either 5 or 25 mM glucose with or without 500 µM palmitate for 72 hours. For in vivo study, we have established a chronic nutrient infusion model in the rat. Serial blood samples were collected for 2 hours after the consumption of a mixed meal to evaluate insulin sensitivity and β-cell function.

Results Chronic glucolipotoxic conditions decreased GLP-1 secretion and the expressions of pCREB, pS6K3, β-catenin, and TCF7L2 in NCI-H716 cells. Glucolipotoxicity conditions reduced glucose transporter expression, glucose uptake, and nicotinamide-adenine dinucleotide phosphate (NADPH) levels in L-cells, and increased triglyceride accumulation. In contrast, PPARα and ATP levels were reduced, which correlated well with decreased levels of SUR1 and Kir6.2, cAMP contents and expressions of pCAMK2, EPAC and PKA. We also observed an increase in reactive oxygen species production, and UCP2 expression and Complex I activity. Simultaneous treatment with insulin restored the GLP-1 secretion. Glucolipotoxic conditions decreased insulin secretion in a time-dependent manner in INS-1 cells, which was recovered with exendin-4 cotreatment. Glucose and SMAC/f lipid infusion for 6 hours decreased GLP-1 secretion and proglucagon mRNA levels as well as impaired the glucose tolerance, insulin and C-peptide secretion in rats.

Conclusion These results provide evidence for the first time that glucolipotoxicity could affect GLP-1 secretion through changes in glucose and lipid metabolism, gene expressions, and proglucagon biosynthesis and suggest the interrelationship between glucolipotoxicities of L-cells and β-cells which develops earlier than that of L-cells.

INTRODUCTION

The primary goal of the treatment of type 2 diabetes mellitus is to control hyperglycemia. However, treatment with conventional therapies such as insulin or insulinotropic sulphonylureas, while useful in reducing hyperglycemia, may impose a higher risk of hypoglycemia.1 Glucagon-like peptide-1-(7-36) amide (GLP-1) is an incretin hormone derived from proglucagon, which is released from intestinal L-cells and stimulates insulin secretion in a glucose-dependent manner, insulin gene expression, and β-cell growth and differentiation.2,3 Evidence that L-cells are themselves nutrient sensitive supports the idea that at least a component of oral glucose-stimulated GLP-1 release arises from the direct stimulation of L-cells by luminal sugars.4 However, GLP-1 is rapidly degraded by the action of the enzyme dipeptidyl peptidase-4 (DPP-4). Therefore, DPP-4 inhibitors and long-acting GLP-1 analogues are widely used to treat people with type 2 diabetes.5 The incretin-based therapies improve glycemic control with a low risk of hypoglycemia and can also have beneficial non-glycemic effects, such as the avoidance of weight gain, reduced blood pressure and improvements in β-cell function.6,6

Glucolipotoxicity might play an essential role in the β-cell decompensation during the development of obesity-associated type 2 diabetes, since the effect of postprandial and subsequently persistent hyperglycemia, added to the high levels of non-esterified fatty acids and triglycerides, could lead to β-cell exhaustion. The secretory machinery of intestinal L-cells shares similarities with that of pancreatic β-cells.6,6 Notably, studies have demonstrated that glucose-stimulated GLP-1
secretion incorporates potassium (K<sub>ATP</sub>) channel and voltage-dependent calcium channels. The precise mechanism by which macronutrients cause incretin hormone release is not fully understood, but direct interaction between the ingested nutrients and the L-cells may be the principal mechanism involved. It has been known from meta-analysis that GLP-1 secretion is not significantly decreased in patients with type 2 diabetes, and although glucolipotoxicity of β-cells is a well-accepted concept, it has not been known whether GLP-1 secretion and/or production is decreased in glucolipotoxic conditions of L-cells. Until now, insulin-secreting β-cells have been thought to be directly affected by glucolipotoxicity.

If glucotoxicity and/or lipotoxicity develop in intestinal L-cells that secrete GLP-1, insulin secretion would also be decreased, and it becomes difficult to reduce hyperglycemia. However, in contrast, if the glucolipotoxicity of the β-cells may preclude it, it may affect the GLP-1 secretion of the L-cell function, as insulin is a well-known GLP-1 secretagogue.

In this study, we investigated whether glucolipotoxicity could be induced by exposure to high glucose and/or fatty acids in L-cells. In vivo experiments were also conducted to investigate the changes of GLP-1, insulin, and C-peptide during prolonged hyperglycemia and/or lipid infusion. To the best of our knowledge, this is the first report on the mechanisms underlying glucolipotoxicity-induced GLP-1 secretory defects from both in vitro and in vivo experiments.

**Materials and Methods**

**Cell culture**

Human NCI-H716 cells (ATCC, Manassas, Virginia, USA) were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) and 1% antibiotics in 5% CO<sub>2</sub> at 37°C. The cells were grown in suspension, so dishes were coated with Matrigel (Becton Dickinson, Bedford, MA), as described previously. Rat pancreatic β-cell line, INS-1 cells, were cultured in RPMI-1640 medium containing 11 mM glucose and supplemented with 10% FBS, 1 mM sodium pyruvate, 10 µM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (pH 7.4), 2.05 mM L-glutamine, and 50 µM β-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin. Glucose was prepared and filter sterilized before use. Palmitate was dissolved in 50% ethanol and coupled to 5% fatty acid-free bovine serum albumin (BSA) at 37°C for 1 hour. All chemicals used in cell culture were obtained from Sigma-Aldrich (Sigma, St. Louis, Missouri, USA) unless otherwise indicated.

**Measurement of GLP-1 secretion**

To determine the responses to glucotoxic and lipotoxic conditions, the cells were exposed to media containing either five or 25 mM glucose with or without 500 µM palmitate for up to 72 hours incubated in Krebs-Ringer bicarbonate-HEPES buffer (140 mM/L NaCl, 3.6 mM/L KCl, 0.5 mM/L NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM/L MgSO<sub>4</sub>, 1.5 mM/L CaCl<sub>2</sub>, 2 mM/L NaHCO<sub>3</sub>, 10 mM/L HEPES) supplemented with 3.3 mM glucose (basal glucose, BG) and 0.1% BSA at 37°C for 1 h under 95% O<sub>2</sub>/5% CO<sub>2</sub> atmosphere for preincubation. The incubation of the cells was performed with Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 3.3 or 16.7 mM glucose (high glucose, HG) for 1 hour at 37°C, and then the medium was collected for detection of total GLP-1 secretion using an enzyme immunoassay (Alpco diagnostics, Salem, NH).

**Western blot analysis**

Cells were lysed with radioimmunoprecipitation assay buffer (RIPA) buffer containing 150 mM NaCl, 20 mM Tris, 0.1% SDS, 1% Triton X-100, 0.25% Na-deoxycholate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 2 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich). Total protein concentration was determined by Bio-Rad DC protein assay, using BSA as a standard (Bio-Rad Laboratories, Hercules, California, USA). Protein was resolved on an sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane, and probed with specific antibodies. The signaling was visualized with ECL Plus Western Blotting Detection System (GE Healthcare, Madison, Wisconsin, USA).

**Total RNA extraction and RT-PCR**

Total RNA was extracted by TRIzol reagent (Invitrogen), and used to prepare cDNA using the Preamplification System of the SUPERSCRIPT First-strand Synthesis System (Invitrogen). The cDNA obtained was amplified directly by PCR.

**Apoptosis assay**

At 24, 48, and 72 hours, cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions.

**Biochemical analyses**

Glucose uptake (BioVision, Milpitas, California, USA), nicotinamide-adenine dinucleotide phosphate (NADPH) (BioVision), triglycerides (TG) (Asan Pharm., Seoul, South Korea), ATP (Promega, Madison, Wisconsin, USA), CAMP (Enzo Life Sciences, Farmingdale, New York, USA), ceramide (MyBioSource, San Diego, California, USA), Complex I activity (BioVision), and insulin (Merodia, Uppsala, Sweden) were measured according to the manufacturer’s directions, respectively.

**Measurement of intracellular reactive oxygen species (ROS) levels and antioxidant enzymes**

The quantification of intracellular ROS levels was carried out using the fluorescent probe, 2,7-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, Oregon, USA) and the carboxy-DCF fluorescence in the cell lysates was measured using a multimode microplate reader (Bio-Rad, Hercules, California, USA) and...
excitation and emission wavelengths of 488 and 530 nm, respectively. Superoxide dismutase (SOD), catalase, and glutathione (BioVision) were determined according to the manufacturer’s directions.

Animal experiments
This study was reviewed and approved by the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Institute (SBRI No. OTC1190171, Seoul, South Korea). SBRI is an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility and abide by the Institute of Laboratory Animal Resources guide. Male Sprague Dawley rats ( OrientBio, Seoul, South Korea) weighing 250–300 g were housed under controlled temperature 21°C and 12 hours light/dark cycle with ad libitum access to water and standard laboratory chow. Under general anesthesia with isoflurane and nitrous oxide, indwelling catheters were inserted into the left carotid artery and right jugular vein. The catheters were tunneled subcutaneously and exteriorized at the base of the neck. The animals were recovered for 7 days after surgery. Catheter patency was maintained with 50 units/mL heparin in 0.9% saline ( Dai Han Pharm., Ansan, South Korea). The animals were randomized into four groups, receiving either 0.9% saline, 50% glucose (D50, Dai Han Pharm), 20% SMOFlipid (IL20, Fresenius Kabi South Korea), or glucose plus SMOFlipid with heparin (20 units/mL), or glucose plus SMOFlipid with heparin. The infusion profile was controlled by a Harvard infusion pump (Pump 33; Harvard Apparatus Canada, Saint-Laurent, QC, Canada) capable of independently operating two syringes simultaneously. Glucose was infused at 2 mL/hour and SMOFlipid at 1 mL/hour for 6 hours. During the infusion, all animals had ad libitum access to food and water, and plasma glucose levels were measured every 2 hours during the infusion period.

Mixed meal tolerance test
After overnight fasting, a mixed meal load (3.7 kcal, 2.5 mL/300 g body weight) was administered orally. The liquid mixed meal consisted of a commercial product (1 can: 300 kcal/200 mL: 12% carbohydrate, 24% protein, 22% fat, Mediwell Diabetic Meal; Mael Dairies, Seoul, South Korea). Blood was drawn by eye bleed at 0, 30, 60, 90, and 120 min after the meal administration for measurement of glucose (AccuCheck II; Roche, Indianapolis, Indy, USA), and plasma samples were stored at −20°C, until further assays. GLP-1 (Alpcos diagnostics), insulin and C-peptide (Mercodia) levels were measured using the corresponding ELISA kits. The insulin sensitivity (Homeostasis model assessment of insulin resistance (HOMA-IR)=fasting glucose×fasting insulin/22.5) and β-cell function index (Homeostasis model assessment of β-cell function (HOMA-β)=20×fasting insulin (µU/mL)/(fasting glucose (mM/L) – 3.5)) were also calculated.

Statistical analyses
Results were expressed as the mean±SD and were analyzed using SPSS V.10.0 software (SPSS, Chicago, Illinois, USA). Data were validated by analysis of variance, and a p<0.05 as indicated by Duncan’s multiple range test was considered to indicate a statistically significant difference.

RESULTS
Chronic glucolipotoxicity reduced GLP-1 secretion
In the presence of BG, GLP-1 secretion was significantly increased under 5 mM G+500 µM P (5 mM GP), and glucolipotoxicity conditions (25 mM glucose+500 µM palmitate, 25 mM GP), compared with under 5 mM glucose (5 mM G). The 25 mM glucose (25 mM G) significantly increased GLP-1 secretion at 24 and 48 hours, compared with these groups. However, GLP-1 secretion was significantly reduced in the chronic hyperglycemia and glucolipotoxicity conditions at 72 hours. In the presence of HG, GLP-1 secretion was significantly increased under 5 mM GP and 25 mM G, than under 5 mM G at 24, 48, and 72 hours; however, it was considerably reduced under chronic glucolipotoxicity conditions at 72 hours (figure 1A). Western blots revealed significant inhibition of cAMP-response element-binding protein (CREB) phosphorylation, and GSK-3β activity as reflected by the decreased phosphorylation of GSK-3β in chronic glucolipotoxic condition, and downregulation of nuclear β-catenin expression and transcription factor 7-like 2 (TCF7L2) as well as GLP-1 (figure 1B), which are compatible with the decreased WNT/β-catenin signaling that is the critical mechanism of GLP-1 biosynthesis and could lead to decreased GLP-1 production and secretion at 72 hours. Chronic glucolipotoxicity conditions significantly decreased the expression of proglucagon in NCI-H716 cells compared with other groups (figure 1C).

Chronic glucolipotoxicity impaired glucose and fatty acid metabolism and K<sub>cat</sub> and Ca channel
There were no significant differences in cell proliferation between groups (figure 2A). The expressions of sodium-glucose cotransporter 1 (SGLT1) and glucose transporter 2 (GLUT2) were significantly increased in 25 mM G, compared with 5 mM G, but were significantly decreased in 25 mM GP compared with 25 mM G (figure 2B). In line with these results, glucose uptake under 25 mM GP significantly decreased, compared with under 25 mM G (figure 2C). Consistent with these data, NADPH levels were significantly decreased in 25 mM G, compared with 25 mM G confirming a dysfunction in glucose metabolism (figure 2D). Protein levels of the fatty acid transporter, CD36, were significantly increased in lipotoxic condition (5 mM GP) and glucolipotoxic condition (figure 2E). Further, we also found fat metabolism to be impaired under chronic glucolipotoxic condition as seen from the 1.5-fold increase in triglyceride levels (figure 2F). PPARα level was significantly decreased in chronic glucolipotoxic condition than under 5 mM GP (figure 2E); and
cellular ATP level was also significantly reduced in chronic glucolipotoxic conditions than under 25 mM G (figure 2G).

Chronic glucolipotoxic conditions impaired sulfonylurea receptor 1 (SUR1), Kir6.2 expressions (figure 2H), and CAMP (figure 2I) level. Ca2+/calmodulin-dependent protein kinase II (CaMkII) phosphorylation and protein kinase A (PKA) expression were significantly increased in 25 mM G compared with 5 mM G, which, however, were significantly suppressed under chronic glucolipotoxic conditions (figure 2J).

Chronic glucolipotoxicity induced ROS production and reduced antioxidant capacity but reversible chronic glucolipotoxicity by recovery insulin in NCI-H716 cells

As shown in figure 3A, chronic glucolipotoxic conditions were associated with significantly increased ROS production. Ceramide concentrations were increased by chronic glucolipotoxicity, but there was no statistically significant difference between groups (figure 3B). Interestingly, uncoupling protein-2 (UCP2) levels were significantly increased in chronic glucolipotoxic conditions (figure 3C). The activity of reduced nicotinamide adenine dinucleotide (NADH)-dehydrogenase, Complex I was increased by 23% in chronic glucolipotoxic condition, than under 5 mM G (figure 3D). Cell permeable SOD, which metabolizes -O2 into H2O2 showed no difference among groups (figure 3E). However, catalase activity (figure 3F) and glutathione content (figure 3G) were significantly decreased in chronic glucolipotoxic condition, compared with under 5 mM G. Meanwhile, there was no significant difference in GLP-1 secretion after 24 hours recovery time from glucolipotoxicity...
Metabolism

A decreased GLP-1 secretion in glucolipotoxicity conditions was reversible by reverting to RPMI-1640 (growth media) for up to 48 and 72 hours (figure 3I,J). Moreover, the decreased GLP-1 secretion in chronic glucolipotoxicity conditions was prevented after the cotreatment with 100 nM insulin (figure 3K) or NCI-H716 and INS-1 coculture for 72 hours (figure 3L).

Chronic glucolipotoxicity reduced insulin secretion in INS-1 cells

INS-1 cell viability was significantly decreased from 24 hours in glucolipotoxicity condition (figure 4A). Decreased insulin secretion was reversible by reverting to RPMI-1640 (growth media) after 24 hours (figure 4B). However, there was no such effect after 48 and 72 hours (figure 4C,D). Furthermore, insulin contents were significantly reduced by 72 hours, compared with 24 hours, except under 5 mM G (figure 4E). Decreased insulin secretion was prevented after the cotreatment with 100 µM Ex-4 for 72 hours (figure 4F). INS-1 and NCI-H716 cocultured for 72 hours in glucolipotoxic conditions prevented the decrease in insulin secretion as well, and the preventive effect was higher than that of Ex-4 only treatment (figure 4G).

Effects on glycemia, pancreatic β-cell function, and incretin levels after mixed meal load in rats

Rats were divided into four groups, and received saline (NS), 50% dextrose (D50), 20% SMOFlipid (IL20), or...
50% dextrose+20% SMOFlipid (Combination) for 6 hours, respectively (figure 5A). The infusions of NS and IL20 did not result in any significant change in blood glucose from baseline. The administration of D50 and Combination resulted in significant rises in blood glucose from baseline by 171% and 99% at 6 hours, respectively. Area under the curve (AUC) of the Combination was significantly decreased, compared with that of D50 (figure 5B). After recovery time for 16 hours, fasting glucose levels of the D50 and Combination group were still significantly higher than those of NS and IL20. AUC of the Combination was significantly increased, compared with that of NS (figure 5C). During mixed meal load, insulin (figure 5D), GLP-1 (figure 5E), and C-peptide (figure 5F) levels of D50, IL20, and Combination were significantly decreased, compared with those of NS. AUCs were decreased in D50, IL20, and Combination group compared with that of NS, but there was no statistically significant difference between groups (figure 5D–F).
HOMA-IR was increased in D50, IL20, and Combination group compared with that of NS, but there was no statistically significant difference between groups (figure 5G).

HOMA-β was significantly decreased by 32%, 24%, and 58% in D50, IL20, and Combination group (figure 5H). The considerable decreases in the phosphorylated form of GSK-3β, as well as β-catenin, TCF7L2, GLP-1, and proglucagon mRNA levels were noticed in D50, IL20, and Combination, compared with those of NS (figure 5I,J).

**DISCUSSION**

GLP-1, an incretin hormone derived from the transcription of the proglucagon gene, is released from the intestinal endocrine L-cells in response to nutritional stimuli. To investigate the effects of chronic glucolipotoxicity on GLP-1 secretion, we generated glucolipotoxic conditions in human origin NCI-H716 enteroendocrine cells using either 5 or 25 mM glucose with or without 500 µM palmitate for 72 hours (chronic glucolipotoxic conditions that mimic diabetic pathology). For in vivo study, we have established a chronic nutrient infusion model in the rat. In our experiments, the glucose and lipids were administered into the vascular spaces, and the lipids, after lipolysis in the intestinal capillaries, could be taken up by the L-cells. The glucose in the vascular spaces could not be taken up by the SGLT1 in the luminal surfaces in the intestinal L-cells; however, it could potentially be taken up by the GLUT2 in the basal surfaces of the intestinal
L-cells in case of glucolipotoxic conditions. Serial blood samples were collected for 2 hours after the consumption of a mixed meal to measure fasting and postprandial glucose, insulin, and C-peptide levels to evaluate insulin sensitivity and β-cell function. GLP-1 secretion was significantly decreased in chronic glucolipotoxic conditions. Infusions of D50, IL20, and combination for 6 hours caused insulin resistance and impaired β-cell function. The functional defects observed in these glucolipotoxic conditions involved coordinated inhibition of glucose-induced GLP-1 secretion, proglucagon biosynthesis, and GLP-1 related protein expressions. Rask et al1 demonstrated in a cohort of normal male subjects with varying degrees of insulin sensitivity that insulin resistance is negatively correlated with GLP-1 secretion. Proglucagon and P1C1/3, an endopeptidase for GLP-1 production from proglucagon, are transcriptionally regulated by CREB. Decreased phosphorylation of GSK-3β and downregulation of β-catenin nuclear expression could, in turn, inhibit GLP-1 secretion. The secretion of active GLP-1 can be regulated at three stages: transcriptional regulation of the proglucagon gene, processing of the proglucagon protein into the active GLP-1, and the exocytosis of the GLP-1 vesicle. TCF7L2 has been known...
to control the transcription of the genes encoding GLP-1 and gastric inhibitory polypeptide (GIP) in the gut and brain. A recent study has revealed that TCF7L2 is critical for the regulation of proglucagon promoter activity. The GSK-3β/β-catenin signaling has been documented to play an important role in TCF7L2 transcriptional activation. Our results indicate that the chronic glucolipotoxicity-induced downregulation of GSK-3β, β-catenin, and TCF7L2 expression might account for the suppression of GLP-1.

There was no significant change in cell viability in chronic glucolipotoxic conditions. SGLT1 and GLUT2 are associated with electrical activity via the closure of K\textsubscript{ATP} channels, and opening of voltage-dependent Ca\textsuperscript{2+} channels. Compared with 5 mM G, the protein levels of glucose transporters were 61% and 57% reduced under chronic glucolipotoxicity conditions, respectively. To determine whether glucose uptake was also affected under chronic glucolipotoxic conditions, we measured glucose uptake into L-cell and observed a ~28% decrease in glucose uptake, indicating that glucose uptake decreased due to impaired expressions of glucose transporters. We could confirm a dominant-negative effect of the glucolipotoxic condition on GLP-1 secretion being regulated through the same glucose-sensing machinery as in β-cells. Consistent with these data, NADPH levels significantly decreased under chronic glucolipotoxic conditions. Intracellular NADPH level increases, when glycolytic precursors or intermediates accumulate, and conditions. Intracellular NADPH level increases, when glycolytic precursors or intermediates accumulate, and glucose-6-phosphate (G6P) is oxidized by G6P dehydrogenase (G6PDH), triggering commitment to the oxidative arm of the pentose phosphate pathway. Increased production of NADPH neutralizes ROS levels. We next ascertained that CD36 and TG were significantly increased, whereas PPARα and ATP content were significantly reduced under chronic glucolipotoxic conditions.

The localization of the K\textsubscript{ATP} channel subunits, SUR1 and Kir6.2, to the gastrointestinal, endocrine L-cells indeed shows that the GLP-1 secretion from these cells is regulated through the same glucose-sensing machinery as in the β-cells. It is of interest that the expressions of the SUR1 and the KCNJ11/Kir6.2 genes, both coding for central parts of the K\textsubscript{ATP} channels, significantly decreased in chronic glucolipotoxic condition. The Zucker Diabetic Fatty rat showed a decreased expression of Kir6.2 mRNA compared with lean control rats, and Kir6.2 mRNA levels dropped by 40% in pancreatectomized, highly hyperglycemic rats, but were nearly unaffected in only mildly hyperglycemic rats. Moritz et al demonstrated that changes in SUR1 transcript levels induced by glucose were reflected by parallel changes in SUR1 protein levels. Recent studies have indicated an association between type 2 diabetes and polymorphisms in these genes as well as in the SLC2A2/GLUT2 gene. The major intracellular functions of cAMP are transduced by PKA and exchange proteins directly activated by cAMP (EPACs). The observation that glucose-induced cAMP signaling is suppressed in palmitate-treated cells is consistent with previous findings that the secretory defect involves a late step in stimulus-secretion coupling. CaMKII has also been suggested to promote Ca\textsuperscript{2+}-dependent intracellular Ca\textsuperscript{2+} release. Increases of cAMP amplify insulin secretion both via PKA and the guanine nucleotide exchange factor Epac. These results suggest that chronic glucolipotoxicity impairs GLP-1 secretion by suppressed cAMP signaling. Chronic glucolipotoxic conditions significantly increased ROS production. Ceramides inhibit the mitochondrial electron transport chain, thereby increasing the generation of ROS. However, there was no significant difference in ceramide levels in chronic glucolipotoxicity condition in our study. Since UCP2 modulates the efficiency of ATP production by catalyzing the translocation of protons across the mitochondrial membrane, one could expect changes in oxidative ATP synthesis. Complex I is the primary electron entry point in the mitochondrial electron transport chain and is the major site for the production of superoxide anion. Superoxide radical, the parental form of intracellular ROS, is a very reactive molecule. It can be converted to hydrogen peroxide by SOD isoenzymes, and then to oxygen and water by several enzymes including catalase and glutathione. In our study, an increase in UCP2 could decrease the ATP production by uncoupling the mitochondrial oxidative phosphorylation, thereby decreasing GLP-1 secretion with associated changes in glucose and lipid metabolism in L-cells.

Glucolipotoxicity contributes to β-cell failure in a time-dependent manner. Insulin secretion was recovered by simultaneous treatment with Ex-4 but not by recovery time. The pancreatic β-cells is a known target of GLP-1 action, releasing insulin in a glucose-dependent fashion. Impaired insulin secretion preceded the decrease in GLP-1 secretion under the same stimulation conditions. GLP-1 secretion appears to be reversible by recovery time and insulin cotreatment. Insulin has been reported to stimulate proglucagon gene expression, as well as GLP-1 synthesis, in GLUTag cells through an Akt-glycogen synthase kinase-3 pathway that involves the bipartite transcription factor, T cell transcription factor-4. In addition, it was possible to discover the association between β-cell toxicity and L-cell toxicity as well as the potential complementarity between them through coculture study. MEK-ERK1/2 pathway was demonstrated to mediate insulin-induced GLP-1 secretion from the L cells and insulin release from the β-cell. We have reported that metformin directly stimulated GLP-1 production and secretion through cross-talk between the insulin and Wnt signaling pathway, in which we also observed the increased expressions of SGLT1 by metformin. It is also very interesting to see that a negative effect of the glucolipotoxic conditions on GLP-1 secretion affected similar mechanisms as in β-cells, and it warrants further study whether the Wnt signaling would also be affected by the glucotoxicity and/or lipotoxicity and whether glucolipotoxicity could be prevented.
by GLP-1 or insulin in vivo, and the pathophysiological implications during the development of type 2 diabetes or insulin in L-cells.

The correlation between microbiota changes and GLP-1 secretion was also reported, and it warrants further study whether the gut microbiota would be affected by the glucolipotoxicity and whether there is any correlation between the changes in gut microbiota and GLP-1 secretion in our model.

Although the clinical trials that the glucolipotoxicity affects GLP-1 were not performed, it was reported that GLP-1 secretion is reduced in patients with type 2 diabetes, and this may contribute in part to the decreased incretin effect and the hyperglycemia that is observed in these individuals. Thus, interest has now focused on the factors that regulate the release of this peptide after nutrient ingestion.

The novelties of our work are that we induced glucolipotoxicity in a nondiabetic animal model, which was associated with the decreased Wnt/β-catenin/TCF7L2 system and failur...
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