Inhibition of cholinergic potentiation of insulin secretion from pancreatic islets by chronic elevation of glucose and fatty acids: Protection by casein kinase 2 inhibitor

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ABSTRACT

Objectives: Chronic hyperlipidemia and hyperglycemia are characteristic features of type 2 diabetes (T2DM) that are thought to cause or contribute to β-cell dysfunction by “glucolipotoxicity.” Previously we have shown that acute treatment of pancreatic islets with fatty acids (FA) decreases acetylcholine-potentiated insulin secretion. This acetylcholine response is mediated by M3 muscarinic receptors, which play a key role in regulating β-cell function. Here we examine whether chronic FA exposure also inhibits acetylcholine-potentiated insulin secretion using mouse and human islets.

Methods: Islets were cultured for 3 or 4 days at different glucose concentration with 0.5 mM palmitic acid (PA) or a 2:1 mixture of PA and oleic acid (OA) at 1% albumin (PA/BSA molar ratio 3.3). Afterwards, the response to glucose and acetylcholine were studied in perifusion experiments.

Results: FA-induced impairment of insulin secretion and Ca2⁺ signaling depended strongly on the glucose concentrations of the culture medium. PA and OA in combination reduced acetylcholine potentiation of insulin secretion more than PA alone, both in mouse and human islets, with no evidence of a protective role of OA. In contrast, lipotoxicity was not observed with islets cultured for 3 days in medium containing less than 1 mM glucose and a mixture of glutamine and leucine (7 mM each). High glucose and FAs reduced endoplasmic reticulum (ER) Ca2⁺ storage capacity; however, preserving ER Ca2⁺ by blocking the IP3 receptor with xestospongion C did not protect islets from glucolipotoxic effects on insulin secretion. In contrast, an inhibitor of casein kinase 2 (CK2) protected the glucose dependent acetylcholine potentiation of insulin secretion in mouse and human islets against glucolipotoxicity.

Conclusions: These results show that chronic FA treatment decreases acetylcholine potentiation of insulin secretion and that this effect is strictly glucose dependent and might involve CK2 phosphorylation of β-cell M3 muscarinic receptors.

Keywords: Pancreatic islets; Fatty acids; Glucolipotoxicity; Acetylcholine; Insulin secretion

1. INTRODUCTION

Neuro-endocrine regulation of the insulin producing pancreatic β-cells is considered a key aspect of the physiology, pathology and pharmacology of glucose homeostasis [14,15,21,36,41]. Numerous neural and hormonal factors are involved in this regulation including the neurotransmitters acetylcholine and nor-epinephrine and the enteric hormones GLP1 (glucagon like peptide 1) and GIP (glucose dependent insulinotropic peptide). Evidence is mounting that defects in the neuro-endocrine regulation of β-cells participate in the molecular pathogenesis of T2DM. For example, GLP1 mediated potentiation of insulin release physiologically associated with food intake is impaired in T2DM [55]. This defect is, at least in part, the result of reduced expression of β-cells GLP1 receptors, which may limit the efficacy of the widely used antidiabetic drugs acting via GLP-1 receptor activation [15,44]. GLP1 receptor loss may be triggered by glucolipotoxicity [50], a process that is known to play a key role in the molecular pathogenesis of T2DM. The concept of “glucolipotoxicity” implies that repeated or continued exposure to high glucose and lipids (specifically to elevated free fatty acids (FAs)) impacting at the same time cause β-cell damage and dysfunction [38,42,43]. The literature data on acetylcholine mediated potentiation of insulin release in obesity and T2DM are very limited [5–7]. Previously, we have shown that FAs interfere acutely with acetylcholine potentiation of glucose stimulated insulin release in studies with isolated mouse islets [13]. It was suggested that this effect of FAs is due to acute emptying of the ER calcium stores,
because it was prevented by inhibition of IP3 receptors [13]. To our knowledge, the chronic effect of glucolipotoxicity on acetylcholine-potentiated insulin secretion in pancreatic islets has not been studied yet.

Although cholinergic regulation of insulin release has been known for many years, the mechanisms by which acetylcholine stimulates insulin secretion are still debated. In their classical paper [2], Ahren and colleagues demonstrated that the non-specific muscarinic receptor agonist carbachol, when administered intravenously, not only potentiated glucose-stimulated insulin secretion in mice fed with either a control or a high-fat diet, but also normalized glucose-stimulated insulin secretion and glucose tolerance in mice subjected to a high-fat diet. This study led to the proposal for the development of islet-muscarinic agonists, with lesser general muscarinic activity, to improve insulin secretion in T2DM [2]. Since then novel muscarinic receptor downstream signaling pathways were discovered which might represent promising new targets for the treatment of T2DM [48]. There are five cholinergic muscarinic receptor subtypes (M₁–M₅), and the work of Gautam and colleagues using transgenic and gene knockout technology have determined that the M₃-muscarinic receptor (M3R) is the main acetylcholine receptor that is responsible for enhancing glucose-dependent insulin release in β cells [18]. The classical mechanism by which M3R regulates insulin release was thought to be primarily via G-protein depending signaling to the calcium and PKC pathways. As a prototypical Gq/11-coupled receptor, activation of M3R induces the hydrolysis of membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP₂), catalyzed by phospholipase C (PLC). This generates two second messengers, inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃, in turn, mobilizes calcium from the IP₃-sensitive stores while DAG activates PKC. Both of these pathways have been thought to play an important role in M3R-mediated insulin secretion [21,48]. However, recent molecular and genetic studies have pointed to further mechanisms. In particular, sustained insulin release associated with the enteric phase appears to be mediated by a process that is independent of G-protein signaling. This is evidenced by studies from the Tobin laboratory and others, which demonstrated that protein kinase D1 (PKD1) is one of the key components by which M3R regulates glucose-dependent insulin release [29,52,53]. M3R is phosphorylated by various kinases, including GPCR kinases and casein kinase 2 (CK2). This process not only leads to the uncoupling of the receptor from its cognate G proteins but also allows for the activation of G protein-independent signaling, a process that is driven largely by the recruitment of β-arrestin adaptor proteins via a G-protein-independent, β-arrestin-dependent process that results in activation of PKD1 and secretary vesicle priming [29]. It is still unclear how G-protein-dependent and independent pathways contribute under different physiological conditions. In particular, the potentiation of GSIR by GPR40 ligands, such as oleic acid [17], occurs due to the G-protein independent pathway [17]. However, recent results from the Wes laboratory showed that M3R in β-cells is specifically phosphorylated by CK2, and this negatively regulates the receptor-mediated insulin release in vitro and in vivo [47]. Preventing M3R phosphorylation, either by inhibiting CK2 in pancreatic β-cells, knocking down CK2z expression, or genetic deletion of CK2z in β-cells of mutant mice, selectively augmented Ca²⁺ signaling and M3R-stimulated insulin release in vitro and in vivo [47]. In support of these results, analysis of human microarray data revealed a significant increase in CK2 expression in human β-cells isolated from T2D subjects, compared with β-cells from nondiabetic donors [32]. It is also possible that receptor phosphorylation by a defined kinase determines a specific signaling outcome and can lead to different physiological effects [53].

Finally, CX4945 treatment protected mice against diet-induced hyperglycemia and glucose intolerance in an M3R-dependent fashion [47]. Based on these data, the goal of the present study was to test whether cholinergic potentiation of insulin secretion is altered due to chronic exposure to elevated glucose and lipids (two major characteristics of T2D) and whether Ca²⁺ signaling or phosphorylation of M3R by CK2 is involved in this effect and whether a selective CK2 inhibitor could prove beneficial under these conditions.

2. RESEARCH DESIGN AND METHODS

2.1. Animals

B6D2SF1 male mice (from Jackson Laboratory) were used throughout. The mice were maintained on a 12:12 h light—dark cycle and were fed a standard rodent chow diet. All research was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (protocol no. 803719).

2.2. Mouse islet: isolation and culture

Mouse islets from male mice were isolated from fed animals using collagenase (EC 3.4.24.3 Serva, 17449) digestion in Hanks buffer followed by separation of islets from exocrine tissue in a Ficoll (Sigma, F-9378) gradient. Isolated islets were cultured for 3—5 days in RPMI 1640 medium (Sigma) containing 10% undialized fetal bovine serum, 10 ml/l penicillin-streptomycin-amphotericin B solution (GIBCO BRL) and 10, 16, or 25 mM glucose with or without free fatty acids noting that undialized bovine albumin contributes variable amounts, but at most 1 mM glucose to the culture medium.

2.3. Human islets: source and culture

Human islets were received from the accredited Human Islet Resource Center at the University of Pennsylvania. The pancreata were procured individually and the isolation performed according to previously described protocols [10,11]. Altogether, we studied pancreatic islets isolated from 18 individuals, all were normoglycemic at organ isolation and ranged in age from 19 to 51 years. Isolated human islets were kept under culture conditions in medium at 5 mM glucose and 25 °C before they were transferred to our laboratory and cultured the same way as mouse islets. Time elapsed from the isolation to transferring the tissue to our laboratory ranged from 1 to 3 days. The culture conditions were similar to those for mouse islets.

2.4. Preparation of fatty acid solution

A 5 mM stock solution of sodium palmitate (Sigma—Aldrich) was prepared by dissolving the fatty acid salt in 10% of bovine serum albumin (BSA, Sigma—Aldrich, fraction V, fatty acid free) in Krebs buffer by continuous stirring for ~ 4 h in a 37 °C water bath. In case of a mixture of palmitate and oleate (2:1; final concentration 5 mM), the sodium palmitate was dissolved first and then sodium oleate was added to the stock solution of 10% BSA. The stock solution was then diluted by Krebs buffer to obtain the final concentration of 0.5 mM sodium palmitate or 0.5 mM of a mixture of palmitate and oleate and 1% of BSA (a molar FA/BSA ratio of 3.3 as seen in extremes of fasting or in diabetic states).

2.5. Perfusion of islets for measurement of insulin release

Cultured islets (130 islets) were placed on a nylon filter in a plastic perfusion chamber (Millipore, Bedford, MA) and were perfused with a flow rate of 1.5 ml per min. The perfusion apparatus consisted of a computer-controlled low pressure chromatography system (Bio-RAD Econo system) that allowed programmable rates of flow and glucose concentration in the perfusate, a water bath (37 °C), and fraction
2.6. Islet batch incubation studies
Islets were isolated as above, hand-picked, and cultured for 3 or 5 days. Batches of 50 islets were loaded into 12 × 75 mm disposable glass culture tubes and preincubated in oxygenated glucose-free Krebs–Ringer bicarbonate buffer at 37 °C for 40 min, followed by a 45 min exposure to different stimuli. After incubations, islets and incubation medium were transferred to 1.5 microcentrifuge tubes and spun at low speed. The supernatant was used for insulin and proinsulin measurements. Pelleted islets were washed two times with cold glucose-free Hank’s buffer and then homogenized with 100 μl of 1% Triton—EDTA buffer.

2.7. Ca2+ measurement
Mouse islets were loaded with fura-2AM during a 40 min pretreatment at 37 °C in 2 ml KRBB supplemented with 5 mM lura-2 acetoxymethylester (Molecular Probes, Eugene, OR). The loaded islets were transferred to the perfusion chamber and placed on the homoeothermic platform of an inverted Zeiss microscope. Islets were perfused with KRBB at 37 °C at a flow rate 1 ml/min, while various treatments were applied to the islets. The intracellular Ca2+ was measured by dual wavelength fluorescence microscopy using a Zeiss AxioVision system as described previously [13,30].

2.8. Insulin measurements
Insulin and proinsulin in islet extracts, culture medium or in the perfusion effluent was measured by radioimmunoassay [35,54].

2.9. Gene expression
Mouse islets were cultured for 3 days with 10 mM glucose, 16.7 mM glucose or 16.7 mM glucose plus 0.5 mM of mixture of palmitate and oleate (2:1). RNA was extracted using the Trizol (Invitrogen) method. The quantitative real time PCR (Applied Biosystems SYBR Green Master Mix kit) were used to explore the expression of selected genes: Csnk2a1 (forward: CATGGAGCACCCTTACTTCTAC, reverse: CTGGGAACAGGTATCCCAAGTG); Csnk2a2 (forward: CTCACCAAGATCTGACTGACT, reverse: GCTCCACATGTCCAAGCTATAA); Caspase3 (forward: TCTGCACTGCTGATTGAA; reverse: GCTCCACATGTCCAAGCTATAA); Bad (forward: CGTCCCTGCTGATCGACTGGAAAGCCGAAAC, reverse: CTGCAAAGGGACTGGATGAA); Akt (forward: CATGGAGCACCCTTACTTCTAC, reverse: CTGGGAACAGGTATCCCAAGTG); and the second phase of insulin release even more (Figure 1D and E), demonstrating again that moderately high glucose per se is not toxic to β-cells under these conditions. To the contrary, β-cell adapt to the increased glucose level. The detrimental effect of PA was, however, clearly evident when glucose in the culture medium was increased to 16 mM (Figure 1D—F): a 50% reduction of glucose-stimulated insulin release and marked right shift of the dose response curve as well as a decrease of maximal acetylcholine potentiation of insulin secretion were seen. A marked decrease in insulin secretion after 3 days exposure to PA and 16 mM glucose was also observed with a glucose ramp (from 0 to 30 mM; applied for a period of 40 min; data not shown). At 25 mM glucose in the culture medium, islets lacked a clear first phase of glucose-stimulated insulin release, a sign of “glucolipotoxicity” (Figure 1G—I). The right shift of the dose response curve and reduced maximal release rate of the acetylcholine-stimulated insulin secretion due to glucolipotoxicity was very pronounced at 25 mM glucose. It is noteworthy that depolarization with 30 mM KCl in the absence of glucose increased insulin release to the same extent in all experiments (Figure 1A,D and G) indicating that basic electrical stimulus-secretion coupling is not affected by these glucolipotoxic conditions.

It was reported that the monounsaturated oleic acid (OA) protects islets from abnormal lipid partitioning, endoplasmic reticulum (ER) expansion and stress caused by saturated FAs [27,37,40]. This prompted us to investigate insulin secretion after islets had been cultured with a mixture of PA and OA at a ratio 2:1 (total concentration 0.5 mM) and two different glucose concentrations: 10 and 16 mM. OA preserved glucose-stimulated insulin secretion in islets cultured at 10 mM glucose (Figure 2A) but not in islets exposed to 16 mM glucose in culture (Figure 2C). Acetylcholine-potentiated insulin secretion was already reduced in islets cultured at 10 mM glucose + PA/OA (Figure 2A; p = 0.02; based on calculation of areas under curves), and it was greatly impaired in islets cultured at 16 mM glucose + PA/OA (Figure 2C) with no evidence for protection by OA. In fact, lipotoxicity seemed more pronounced on acetylcholine potentiation of insulin release with the mixture than with PA alone (compare Figure 1A and C with Figure 2A and C, respectively). In order to test whether KATP channels might be the step primarily targeted by FA toxicity, we performed experiments with the channel

0.5 mM palmitic acid (PA) at 1% (PA/BSA molar ratio 3:3) in RPMI culture medium containing 10% of bovine serum albumin at 10, 16, or 25 mM glucose. The effects of acetylcholine on insulin release were tested in the presence of either glucose, an amino acid mixture or α-ketosioacapric acid (KIC). Acetylcholine augments the response to 8 mM glucose by 8—10 fold [13]. This test condition provides an excellent, extremely sensitive way for studying β-cell secretory responsiveness and capacity and for detecting functional defects of pancreatic islets. The effects of PA on insulin secretion were already manifest at 10 mM glucose (Figure 1A—C), the glucose concentration routinely required for culturing rodent islets and maintain glucose and acetylcholine responsiveness. An increase in glucose concentration from 4 to 8 mM caused a biphasic increase in insulin secretion in the functional test using an islet perfusion protocol. The first and second phases were decreased in PA-treated islets (Figure 1B). A gradual increase in acetylcholine concentration from 0 to 1 μM in the presence of 8 mM glucose led to a marked potentiation of hormone release in both groups of islets. However PA-treated islets exhibited a right shift of the dose response curve (Figure 1C). Culturing at 16 mM glucose without FA addition increased first phase insulin secretion about 2 fold and the second phase of insulin release even more (Figure 1D and E), demonstrating again that moderately high glucose per se is not toxic to β-cells under these conditions. To the contrary, β-cell adapt to the increased glucose level. The detrimental effect of PA was, however, clearly evident when glucose in the culture medium was increased to 16 mM (Figure 1D—F): a 50% reduction of glucose-stimulated insulin release and marked right shift of the dose response curve as well as a decrease of maximal acetylcholine potentiation of insulin secretion were seen. A marked decrease in insulin secretion after 3 days exposure to PA and 16 mM glucose was also observed with a glucose ramp (from 0 to 30 mM; applied for a period of 40 min; data not shown). At 25 mM glucose in the culture medium, islets lacked a clear first phase of glucose-stimulated insulin release, a sign of “glucolipotoxicity” (Figure 1G—I). The right shift of the dose response curve and reduced maximal release rate of the acetylcholine-stimulated insulin secretion due to glucolipotoxicity was very pronounced at 25 mM glucose. It is noteworthy that depolarization with 30 mM KCl in the absence of glucose increased insulin release to the same extent in all experiments (Figure 1A,D and G) indicating that basic electrical stimulus-secretion coupling is not affected by these glucolipotoxic conditions.

3. RESULTS
3.1. Cholinergic potentiation of insulin secretion in mouse islets is impaired by glucolipotoxicity
Our experiments on the “glucolipotoxicity” phenomenon were performed with isolated islets in organ culture attempting to mimic the in vivo conditions: pancreatic islets were cultured for 3 days with

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inhibitor glyburide. A gradual increase of glyburide (12.5 nM increment/min) in the presence of a low glucose concentration (3 mM) induced comparable stimulation of insulin release in control and experimental groups of islets (Figure 3). Following saturation with glyburide at 1 mM, high glucose (16 mM) was added on top of glyburide for an additional 20 min. High glucose was much less effective in the FA-treated islets (Figure 3). These data suggest that glucose amplification of insulin secretion rather than the triggering by a K-channel blocker is impaired by FA treatment, pointing to a site of action upstream in stimulus-secretion coupling. This notion is consistent with the results and interpretation of β-cell depolarization with KCl (see Figure 1A, D and G).

**Figure 1:** Effects of palmitic acid on glucose- and acetylcholine-stimulated insulin release in mouse islets. Islets were cultured for 3 days with or without 0.5 mM palmitate (bound to 1% BSA) and increasing concentrations of glucose: 10 mM (Panel A); 16 mM (Panel D); 25 mM (Panel G). Panels B and C, E and F, H and I: a magnified view of selected sections of the perfusion experiments from Panels A, D and G is presented to clearly show the inhibiting effect of fatty acids on glucose and acetylcholine stimulation of insulin secretion. An acetylcholine (Ach) ramp from 0 to 1 μM (12.5 nM increment/min) was applied after a 90 min islet preperfusion with 0, 4, and 8 mM glucose (G) (30 min for each intervention). 8 mM glucose was present during the acetylcholine ramp. Open circles: cultured with glucose alone; filled circles: glucose plus 0.5 mM palmitate. Effect of glucolipotoxicity on insulin secretion in mouse islets strongly depends on glucose concentration. Note: Here and in all other experiments that include acetylcholine, 10 μM neostigmine, a cholinesterase inhibitor, was used to prevent its breakdown. The sign “+” indicates that acetylcholine was added on top of 8 mM glucose. FAs are not present during the test. Each curve represents the mean ± SE of 3–4 perfusions.
3.2. Absolute glucose requirement for pancreatic β-cell lipotoxicity
both in the culture medium and in the test system used for demonstrating lipotoxicity

There is compelling evidence that FAs are toxic in pancreatic β-cells only in the presence of unphysiologically high glucose [38,42,43]. A compelling molecular explanation for this glucose requirement is still not available. It was suggested earlier that glucose toxicity could be related to generation of ceramide as has been shown in human islets [31]. Glucose is commonly considered a critical, irreplaceable fuel substrate in islet cell culture for maintaining optimal energy metabolism, insulin biosynthesis, and stimulus-secretion coupling for stimulated insulin secretion. We had demonstrated earlier, however, that islets preserve full structural and functional integrity for at least one week when glucose in culture medium is replaced by a mixture of glutamine and leucine (7 mM each) [34,56]. Such a culture system promised to be well suited for studying the molecular basis of the absolute glucose requirement for β-cell lipotoxicity. Note, in the present studies, the fetal calf serum added to the culture medium was not dialyzed in contrast to the cited reports so that a variable glucose contamination of less than 1 mM was still present. Two experimental designs were used: 1) high glucose (10–25 mM) in culture medium was replaced by 7 mM glutamine plus 7 mM leucine, as alternate substrates supporting islets survival and functionality in the presence and absence of FA, and islets were then tested in perfusion experiments with glucose plus leucine as fuel stimulants; 2) islets were cultured with glucose (16 mM) but then glucose in the perfusion test system was replaced with 4 mM of a physiological amino acid mixture or KIC for functional tests of the islets. In the first scenario, the acetylcholine potentiation of insulin secretion was fully preserved when the response to acetylcholine was tested in the presence of 4 mM glutamine plus 10 mM leucine instead of 10 mM glucose (Figure 4A). In the second design, acetylcholine potentiation of insulin secretion was also preserved when islets were cultured under glucolipotoxic conditions, i.e. with high glucose and FAs, but tested with a 4 mM amino acid mixture (Figure 4B) or 8 mM KIC (Figure 4C). According to these exploratory studies, such very low glucose culturing and glucose free test systems provide unique experimental opportunities for the precise manipulation of glucose metabolism and for uncovering critical glucose-derived metabolites or processes for lipotoxicity to occur. It remains to be critically tested whether the very low glucose contamination in the culture medium makes any difference. It is very unlikely that glucose level below 1 mM is effective because of the characteristics of glucokinase, the critical step of glucose metabolism in pancreatic β-cells [33].
3.3. Islet insulin content and insulin release decreased during culture under glucolipotoxic conditions

The islet insulin content was reduced by half after 3 days, yet less so after 5, in culture with the FA mixture at 10 or 16 mM glucose (Table 1). Proinsulin content was also reduced by about 50% in fatty acid-treated islets resulting in unchanged proinsulin/insulin ratios. The cumulative insulin release measured in the culture medium after 3 days of exposure to fatty acids was slightly elevated at 10 mM but not at 16 mM glucose (Table 1). The results show that islet insulin/pro-insulin stores under glucolipotoxic culture conditions are markedly reduced, suggesting that the results of acute functional tests in a perfusion system might be influenced by this deficit. The total insulin release data shown in Table 1, however, are more difficult to interpret because the time course of the secretion profile is not sufficiently known. The reduced insulin content could be due to β-cell death. Therefore, we measured the mRNA expression of the genes involved in cell apoptosis. We found that the expression of apoptotic gene Caspase3 is increased in islets exposed to 16 mM glucose plus 0.5 mM PA/OA mixture compared to glucose alone (Figure 5B). At the same time, the expression of mitochondrial pro-apoptotic gene Bad and anti-apoptotic gene Bcl-XL was unchanged in these samples. High glucose alone did not change the expressions of these genes (Figure 5A). The results shown in Figure 4B and C, however, suggest that reduced insulin stores or an increase in the expression of Caspase3 after 3 day culture have no impact on insulin secretion. It may take a longer time to see the changes in insulin secretion in our acute functional tests. In fact, if insulin release were expressed as a fraction of insulin content, responsiveness to stimulant would appear unchanged or enhanced as result of glucolipotoxic treatment. In view of the extensive literature on the molecular mechanisms of...
Table 1 — Mouse islet insulin and proinsulin content and total insulin release after 3 and 5 days culture with or without a mixture of PA and OA (0.5 mM) and with two different glucose concentration (10 and 16 mM).

| Insulin/Proinsulin | Insulin content, ng per islet | Proinsulin content, ng per islet | Proinsulin/Insulin ratio | Total insulin release, ng per islet | Total insulin, ng per islet | Total insulin, ng per islet |
|--------------------|-------------------------------|---------------------------------|--------------------------|-----------------------------------|---------------------------|---------------------------|
| Culture conditions | 3 days                        | 5 days                          | 3 days                   | 5 days                            | 3 days                    | 5 days                    |
| G, 10 mM           | 50.5 ± 1.8                    | 56.6 ± 1.6                      | 44.2 ± 4.4               | 45.1 ± 5.4                        | 0.87                      | 0.80                      |
|                    | G, 10 mM + PA + OA            | 29.6 ± 3.3*                    | 39.5 ± 3.4*              | 23.1 ± 3.9*                       | 24.2 ± 4.8*               | 0.78                      | 0.61                      |
|                    | G, 16 mM                      | 47.0 ± 3.3                      | 43.8 ± 2.4               | 44.2 ± 2.3                        | 45.5 ± 6.3               | 0.94                      | 1.04                      |
|                    | G, 16 mM + PA + OA            | 24.3 ± 3.9*                    | 32.5 ± 2.5*              | 19.6 ± 2.5*                       | 28.4 ± 3.6*              | 0.81                      | 0.87                      |

Insulin and proinsulin content was measured by harvesting 10 islets at the end of 3 or 5 days of culture in RPMI 1640 medium containing 10 or 16 mM glucose ± a mixture of PA and OA. Total insulin released was measured in the incubation supernatants using a radioimmunoassay available at the RIA Core in the Diabetes Center at the University of Pennsylvania Perelman School of Medicine. Results are presented as means ± SE of 4 experiments. Note: The total insulin pool per islet included also proinsulin measured in islets. * p < 0.05 compared to culture with the same glucose level but without fatty acids present.

Figure 5: Gene expression profile of mouse islets. Panel A: Comparison of relative mRNA expression of CK2α1 (Csnk2a1), Caspase3, Bad and Bcl-XL in islets exposed for 3 days to 10 and 16 mM glucose (n = 4). Panel B: Effect of fatty acids (0.5 mM PA/OA) on gene expression at 16 mM glucose (n = 10).
glucolipotoxicity for insulin biosynthesis showing uniformly marked inhibition of insulin production after exposure to glucolipotoxic condition, the present results are considered to be in agreement [42].

3.4. Glucolipotoxicity reduces acetylcholine-stimulated Ca\(^{2+}\) release from the ER

IP\(_3\)-R mediated Ca\(^{2+}\) release from the ER participates in glucose- and acetylcholine-stimulated insulin secretion [21]. To test whether acetylcholine-stimulated Ca\(^{2+}\) release from the ER is affected by glucolipotoxicity, mouse islets were stimulated first with 10 mM glucose and then 10 \(\mu\)M nifedipine was added to inhibit the voltage dependent calcium channel and Ca\(^{2+}\) entry from extracellular medium. Afterwards, 1 \(\mu\)M of acetylcholine was applied to stimulate Ca\(^{2+}\) release from the ER. Both glucose- and acetylcholine-stimulated Ca\(^{2+}\) release were reduced in islets exposed for 3 days to 16 mM glucose and to 0.5 mM of a mixture of PA and OA acid (Figure 6A). The area under the curve indicates the significance of the changes in glucose- and acetylcholine-stimulated Ca\(^{2+}\) signaling (Figure 6B and C).

3.5. Preserving ER Ca\(^{2+}\) stores by inhibition of the IP\(_3\) receptor (IP3R) does not protect islets from glucolipotoxicity

The reduced acetylcholine-stimulated Ca\(^{2+}\) release from ER may be related to decreased ER calcium storage capacity due to chronic exposure to FAs. It has been shown that PA causes a decrease in ER calcium storage capacity as measured by thapsigargin-induced Ca\(^{2+}\) release [9] or measured directly with the fluorescent protein-based ER Ca\(^{2+}\) sensor D1ER [23] without concomitant measurement of insulin secretion. In this study, we revaluated the effects of FAs on ER calcium release in order to determine their involvement in reduced acetylcholine-stimulated insulin secretion. We used thapsigargin to estimate the calcium storage capacity of ER. It is noteworthy that culturing at 16 or 25 mM glucose alone led to a decrease in maximal glucose-stimulated and also of thapsigargin-induced Ca\(^{2+}\) rise in mouse islets (Figure 7A and B). In addition, increasing the glucose concentration in culture medium left shifted the glucose—dependency curve of the Ca\(^{2+}\) response (Figure 7B). The effect of PA on glucose and thapsigargin-stimulated Ca\(^{2+}\) release was studied in islets cultured at 10 mM glucose ± 0.5 mM PA (higher glucose of 16 and 25 mM alone eliminated the thapsigargin effect), PA marginally decreased the Ca\(^{2+}\) response to 10 mM glucose and completely eliminated the thapsigargin-induced Ca\(^{2+}\) release (Figure 7C).

Previously, we showed that blocking IP\(_3\)-Rs of ER completely abolished the acute inhibitory effect of PA on glucose dependent acetylcholine potentiation of insulin secretion [13], suggesting a role of the IP\(_3\)-R in the inhibitory effect of FAs. To test whether IP\(_3\)-Rs are similarly involved in the harmful effects of PA on insulin release and changes in Ca\(^{2+}\) signaling during chronic exposure to FAs, the highly specific inhibitor of the IP\(_3\)-R xestospongin C was added to the culture medium. IP\(_3\)-R inhibitor appeared to preserve a relatively small thapsigargin effect in palmitate-exposed islets (Figure 7D) but did not prevent a decrease in glucose stimulated and acetylcholine potentiated insulin release (Figure 7E) (comparable to the results presented in Figure 1D). The glucose-induced Ca\(^{2+}\) rise was also diminished in PA treated islets.

3.6. Reversing the effect of glucolipotoxicity on insulin secretion by CK2 inhibition

It was recently discovered that CK2 phosphorylation of β-cell M3Rs modulates the activity of this receptor in pancreatic β-cells and that inhibition of CK2 in vivo protects mice from diet-induced hyperglycemia [47]. Here we tested whether CK2 mediated phosphorylation is possibly involved in inhibition of M3Rs and acetylcholine-potentiation of insulin secretion in β-cell exposed to glucolipotoxicity in culture. Treatment of pancreatic islets in culture with the highly selective CK2 inhibitor CX4945 completely preserved insulin secretion when islets were exposed to less severe glucolipotoxicity with 10 mM glucose (Figure 2B; Compare to Figure 2A) and partially protected acetylcholine-potentiated insulin secretion in mouse islets cultured for 3 days at 16 mM glucose + 0.5 mM PA/OA mixture (Figure 2D; Compare to Figure 2C). It was reported that the expression of CK2α (Csnk2a1) is moderately increased in human islets isolated from T2D...
Figure 7: IP$_3$R inhibitor Xestospongin C did not prevent the inhibitory effect of palmitic acid on insulin release (Panel A) and on the glucose-dependent Ca$^{2+}$ rise (Panel B). Panel A: Effects of islet culture for 3 days at different glucose concentrations on glucose- and thapsigargin-stimulated Ca$^{2+}$ rise. Typical experiment is presented ($n = 4$). Panel B: A magnified view of selected section of the perfusion experiment from Panel A to clearly show the left shift and decrease maximum of the glucose-stimulated calcium rise. Panel C: Intracellular Ca$^{2+}$ signaling after islet culturing for 3 days with 0.5 mM PA and 10 mM glucose. Typical experiment is presented ($n = 4$). Panel D: Islets were cultured for 3 days with 16 mM glucose ± PA (0.5 mM) and in the presence of IP$_3$R inhibitor Xestospongin C (1 μM). Panel E: Insulin secretion profile for the experiments presented on panel D. The perfusion was done in the absence of the inhibitor (compare with panel D of Figure 1). Data presented as means ± SE of 4 experiments.
Our data showed that the CK2α1 expression (Csnk2a1) did not significantly change due to exposure to high glucose (Figure 5A) or high glucose and PA/OA mixture (Figure 5B).

### 3.7. Effects of fatty acids on isolated normal human islets

When the same experimental design was employed in studies of human islets, insulin secretion stimulated by 4 or 8 mM glucose was found to be decreased or even abolished in all experiments (n = 7) after 3 days exposure to 0.5 mM PA and 16 mM glucose (Figure 8A). It is noteworthy that, quantitatively, insulin release is only 10% of that observed with mouse islets (compare Figures 1 and 8). The 3−4 fold acetylcholine-potentiation of insulin release was unchanged in 3 of 7 experiments while a decrease was seen in the other 4 islet preparations. Still, on average the insulin secretion profile was apparently unchanged (Figure 8A). In another set of experiments, islets were exposed for 3 days to a mixture of 0.5 mM PA and OA and 16 mM glucose, and the responses to consecutive additions of 4 and 8 mM glucose followed by an acetylcholine ramp were tested in perfusion experiments. Based on calculation of areas under curves, insulin release due to stimulation with 4 or 8 mM glucose was reduced by 24% (p = 0.039) and 59% (p = 0.39), respectively. In contrast to the experiments with PA alone, islet culturing with the mixture of PA and OA showed a marked decrease in acetylcholine-potentiated insulin secretion in all 3 experiments (Figure 8B). In a third set of experiments in which the acetylcholine ramp was applied at 6 mM glucose (n = 3), the results indicate that the FA mixture has a greater effect on acetylcholine-stimulated insulin release than PA alone. We have also tested in human islets whether inhibition of CK2 would protect islets from glucolipotoxicity. CX4945, added during the 3-day culture, completely protected acetylcholine-potentiated insulin secretion in all three cases (Figure 8D; see panel B for comparison).

### 4. DISCUSSION

The new contribution of the present study is the demonstration of glucolipotoxic impairment of acetylcholine potentiation of GSIS with a well-controlled tissue culture model system using isolated normal mouse and human pancreatic islets. This demonstration together with the widely accepted view that cholinergic innervation of β-cells participate in their survival and function suggest high translational relevance for the molecular pathogenesis of T2DM and perhaps therapeutic applicability. It is hypothesized that glucolipotoxic impairment of cholinergic regulation of β-cells is comparable in importance to impaired regulation through the β-cell GLP-1 receptor in T2DM. Such defective neuro-endocrine control of pancreatic β-cells is here envisaged as secondary to hyperglycemia together with hyperlipidemia, more specifically to elevated serum FAs, a hallmark of obesity and diabetes. The decompensated disease state develops only in individuals in whom the physiological positive feedback by nutrients (glucose, amino acids and FAs) and the neuro-entero-endocrine system (vagus, GLP-1 and GIP) is defective because of a diabetes prone genetic disposition.

Figure 8: Chronic effects of FAs on isolated human islets in culture. Panel A: Islets were cultured for 3 days in the presence of 16 mM glucose ± 0.5 mM of PA. Glucose-stimulated insulin release was reduced in all experiments. Acetylcholine-potentiation of insulin secretion was unchanged in experiments with 3 isolates but was reduced in another 4 islet preparations resulting in statistically insignificant changes (n = 7). Panel B: Islets were cultured for 3 days with 16 mM glucose ± 2:1 mixture of PA and OA (n = 3). Panel C: Experimental condition was the same as in Panel B except acetylcholine ramp was applied at 6 mM glucose (n = 3). Panel D: Experimental condition was the same as in Panel B except inhibitor of casein kinase 2 was added to culture medium (n = 3).
The results of the present study highlight several distinct aspects of the glucolipotoxicity phenomenon. They show that 1) high levels of glucose (≥10 mM) are absolutely required for inducing as well as demonstrating lipotoxic insulin secretion defects; 2) glucose amplification of insulin secretion rather than the triggering by a K-channel blocker is damaged by FAs pointing to a site of action upstream of this step in stimulus-secretion coupling; 3) in contrast to acute action of FAs [13], preserving the ER Ca²⁺ storage capacity by blocking IP₃-mediated Ca²⁺ release from ER during 3 day culture is not able to prevent glucolipotoxic effects on insulin secretion; 4) in contrast, a specific inhibitor of CK2 completely preserved mouse islet function cultured at 10 mM glucose and partially protected islets from glucolipotoxicity at 16 mM glucose; 5) most importantly, glucolipotoxic impairment of cholinergic potentiation of GSIR is demonstrable with isolated normal human islets and the inhibitor of CK2 completely protected human islets against glucolipotoxicity. These and other aspects are further discussed below.

4.1. Acetylcholine potentiation of fuel stimulated insulin release in isolated mouse islets

Our study demonstrates that acetylcholine potentiation of GSIR is markedly reduced after chronic exposure to FAs. To our knowledge, this is the first report on effects of chronic FA treatment on cholinergic potentiation of fuel induced insulin secretion. This effect strongly depends on the glucose concentration in the culture medium with delayed onset of potentiation already demonstrable at 10 mM glucose and delayed onset plus reduced maximal effectiveness of the neurotransmitters observable at 16 and 25 mM glucose. Cholinergic stimulation enhances insulin secretion and is a critical factor permitting expansion of the pancreatic β-cell mass when needed, thus contributing importantly to maintaining optimal glucose homeostasis [1,2,11]. If FAs do indeed impair cholinergic potentiation of insulin secretion and β-cell replication/survival in vivo, then this effect may contribute to impaired compensation when insulin resistance develops for any reason (e.g. obesity) resulting in T2DM. In support of this view, persistent activation of β-cell M3Rs in transgenic mice (β-M3 Q490L) or β-cell stimulation by clozapine—NO in mice with a redesigned M3R protects against the damage from a high-fat diet [19,26].

Previously, we found that PA acutely decreases acetylcholine potentiation of insulin secretion [13], and this effect was associated, if not caused, by emptying of ER calcium stores and prevented by an IP3R inhibitor. In the present study, we confirmed the findings by others that chronic exposure to FA lead to decreased ER calcium stores as evidenced by a decreased thapsigargin-induced calcium rise in FA-treated islets (see Figure 7C). However, in the case of chronic exposure to FAs, the observed decrease in acetylcholine potentiation of insulin secretion is more likely unrelated to decreased ER Ca²⁺ storage capacity, because FA damage was not prevented by inhibition of IP3Rs (see Figure 7E). Chronic exposure to PA decreases IP3 levels in rat pancreatic islets [51]. It is possible, therefore, that FAs may reduce PLC activation and hydrolysis of IP2 resulting in decreased signaling through M3Rs. The effect of acetylcholine is glucose dependent. The possibility must therefore be considered that the decrease of acetylcholine potentiation of insulin release by FAs is entirely or at least in part the result of impaired glucose-stimulated insulin secretion. This explanation is plausible in view of the results shown in Figure 4. However, the results presented on Figure 2A suggest that cholinergic potentiation of insulin secretion is more sensitive to glucolipotoxic damage than stimulation of hormone release by glucose alone.

4.2. Absolute glucose requirement for eliciting lipotoxicity in the islet culture model

To better understand the apparently crucial contribution of glucose to lipotoxic islet failure, nominally “glucose-free culture and testing systems” were used in present study [34,56]. It is striking that a high concentration of FAs (0.5 mM) did not decrease the insulin secretion after 3 day culture when the standard fuel glucose of at least 10 mM (optimal for culturing rodent islets) was replaced by glutamine and leucine, 7 mM each. It is also remarkable that even 3 day exposure of islets to high 16 mM glucose and FAs did not cause any alteration in insulin secretion when glucose in the test system was replaced by a physiological mixture of amino acids or KIC permitting acetylcholine potentiation. The 4 mM amino acid mixture or KIC may suffice for this purpose because both serve as the ATP generating fuel without producing putative “toxic metabolites” otherwise derived from high glucose. This demonstrates that glucose is needed not only for inducing the lipotoxic effect but also for demonstrating it in the test system. Such glucose-free culturing and functional test systems provide unique experimental opportunities for precise manipulation of glucose metabolism and for uncovering critical glucose-derived metabolites or processes for lipotoxicity to occur. In the present study, this strategy could not be fully exploited because it was applied only late into this investigation. This new strategy has, however, considerable promise to advance our limited understanding of glucolipotoxicity. With its application new pharmacological or nutritional strategies to protect pancreatic islets against lipid-induced β-cell failure may be discovered.

4.3. Arguments against a critical involvement of Kₐ₅₆ channel defects in glucolipotoxicity

Kₐ₅₆ channels determine the responsiveness of pancreatic β-cells to fuel stimulants and their inhibition by an increased P-potential (ATP/ADP + ADP) or sulfonlurea drugs permits neuro-endocrine potentiation (e.g. by acetylcholine or GLP-1) or even potentiation by FA via the GPR40 receptor. Some studies showed that long-chain CoA (LC-CoA) esters serve as activators of Kₐ₅₆ channels in rodent β-cells [3,4,22], and thus appear to reduce the sensitivity of the channel to alterations of the P-potential in a way similar to phosphatidylinositol phosphates [49]. An activating effect of long-chain CoA esters on this channel was also inferred because it is prevented by triacsin C, an inhibitor of long fatty acyl CoA synthase [57]. Based on this information, it was speculated that FA-induced activation of Kₐ₅₆ channels is the primary cause for the inhibition of glucose stimulated insulin secretion by FAs. This proposed mechanism is not compelling for several reasons. The above literature relates only to acute action of FAs on Kₐ₅₆ channels. Yet, FAs acutely stimulate insulin secretion, most likely through activation of GPR40, but do inhibit GSIR only after long-term treatment [24]. Furthermore, the Kₐ₅₆ channel blocker tobutamide failed to stimulate insulin secretion beyond that evoked by 20 mM glucose alone in islets that had been cultured with 15 mM glucose and OA or PA for 72 h [59]. Furthermore, transcript levels for genes encoding Kir6.2 and SUR1 and the resting Kₐ₅₆ channel activity were unaffected by high glucose and FAs [39]. We tested here whether Kₐ₅₆ channels might be involved in inhibition of insulin secretion by chronic exposure to high glucose and FAs using glyburide applied as ramp at low 3 mM glucose. The response of β-cells to the glyburide ramp was not at all influenced by prior FA treatment, suggesting that glucolipotoxic insulin secretion defects are probably related to the amplification signaling pathway.
4.4. FA lowers insulin/proinsulin stores

Results of the present study (Table 1) showed a significant decrease in insulin and proinsulin content in islets cultured at 10 and 16 mM glucose in the presence of PA and OA (0.5 mM). In contrast to content, the total (cumulative) insulin was slightly elevated due to FA at 10 mM glucose but unchanged at 16 mM glucose. The reduced insulin/proinsulin stores due to FA already clearly visible at 10 mM glucose and it can be a very sensitive indicator of glucolipotoxicity. The threshold for inducing glucolipotoxicity may be well within the physiological range of 5–8 mM.

It was reported before that the islet insulin content is reduced due to glucolipotoxicity [39,51]. Chronic glucolipotoxicity also down-regulates insulin gene expression through decreasing Pdx levels. Olofsson et al. [39] showed dissociation between insulin content and decrease in insulin secretion after chronic exposure to different glucose concentrations and OA or PA. Our results agree with Olofsson et al. [39], because stimulated insulin release seems to be independent of the islet store, as illustrated by Figure 4B and C. Chronic exposure to high glucose and FAs may lead to cell death, which also could impact the amount of released insulin. Previously, we showed that the expression of the apoptotic transcription factor C/EBP homologous protein (CHOP) was increased by 20% in mouse and by 95% in human islets due to exposure to 16 mM glucose and 0.5 mM PA for 3 days [12]. Exposure of human islets to a mixture of PA and OA and 16 mM glucose increased CHOP expression by 65% [12]. Results of the present paper showed that the expression of apoptotic gene Caspase3 is increased in islets exposed to 16 mM glucose plus 0.5 mM PA/OA mixture. However, the expression of mitochondrial pro-apoptotic gene Bad and anti-apoptotic gene Bcl-XL was unchanged under these conditions. Again, the results presented in Figure 4 did not confirm that the increased expression of apoptotic genes affects insulin secretion in islets exposed to glucolipotoxic conditions for 3 days.

4.5. FAs impair intracellular Ca\(^{2+}\) signaling

Studying the effects of FA on Ca\(^{2+}\) signaling using routine fluorescence based level measurements of free Ca\(^{2+}\) is made difficult by the complex influence that glucose by itself has on calcium metabolism of pancreatic β-cells (see Figure 7A and B). With increasing glucose in the islet culture phase the dose response curve of glucose stimulated free intracellular Ca\(^{2+}\) shows a left shift, a reduced maximum, and lack of a thapsigargin effect. These changes are probably an expression of adaptation at 10 and 16 mM glucose but could be causally involved with glucotoxicity at 25 mM (see Figure 1 B, E and H). Lipotoxicity to Ca\(^{2+}\) physiology is manifest most clearly at 10 mM glucose. This illustrates that the role of calcium metabolism in the glucolipotoxicity phenomenon needs to be studied at relatively low glucose levels (i.e. between 5 and 10 mM). The reduced calcium response could be related to decreased Ca\(^{2+}\) entry from the extracellular space or decreased Ca\(^{2+}\) mobilization from intracellular stores. Saturated and, to a lesser extent, unsaturated FAs cause β-cell ER stress and apoptosis with chronic exposure [8,9,28] through Ca\(^{2+}\) release from ER and secondary to protein misfolding [9]. Direct measurement of ER Ca\(^{2+}\) release using the fluorescent protein-based ER Ca\(^{2+}\) sensor D1ER [23] showed that PA and to the same extent OA stimulate Ca\(^{2+}\) release from ER. In support of these data, the results of the present study indicate that chronic exposure to PA resulted in reduced calcium storage capacity of ER as measured by thapsigargin-induced Ca\(^{2+}\) release (clearly seen in culture with 10 mM glucose). Thapsigargin blocks ER Ca\(^{2+}\) ATPase and thus increases cytosolic Ca\(^{2+}\). Acutely FAs stimulate ER Ca\(^{2+}\) release by activating IP3 receptors [16,20]. Acetylcholine also stimulates generation of IP3, which causes a rapid mobilization of Ca\(^{2+}\) from ER [21]. Indeed, acetylcholine in the presence of nifedipine increased intracellular Ca\(^{2+}\) levels and this stimulation was reduced in islets exposed to glucolipotoxicity (Figure 6). The latter effect could be due to lower ER calcium capacity or reduced signaling through M3Rs. The inhibition of IP3 receptors by xestospongion during islet culture appeared to preserve the ER Ca\(^{2+}\) storage capacity (Figure 7D) but did not prevent the reduction in insulin secretion caused by FA (Figure 7E). In sum, current information lets one conclude that ER calcium depletion by FA is not the critical event leading to β-cell dysfunction.

4.6. FAs impair acetylcholine activation in human islet in a culture model of glucolipotoxicity

The effects of FAs on human islets were greatly influenced by the culturing and testing conditions, specifically whether PA was used alone or in combination with OA. When human islets were cultured with PA and 16 mM glucose, glucose-stimulated-insulin release was decreased or even abolished after 3 days exposure. However, the response to acetylcholine varied greatly in different experiments, and a decrease in insulin secretion was seen only in 4 of 7 islet preparations. However, when islets were cultured with the mixture of PA and OA, acetylcholine potentiation of insulin secretion was 50% lower in all cases (see Figure 8B and C). Thus, in both mouse and human islets, the impairment in acetylcholine potentiation of insulin secretion was more pronounced when islets cultured with 16 mM glucose were exposed to PA/OA mixture than to PA with no evidence of protection by OA. Studying cholinergic mechanisms in isolated human islets is greatly complicated by the observation that the α-cells appear to contain and release acetylcholine in contrast to rodent α-cells, implying a paracrine stimulatory control system for β-cells [45,46] in parallel to regulation by the vagus nerve. We have neglected this putative paracrine process, which, in our judgment, must be quantitatively minor considering the 3–4-fold increase of insulin release and the very steep DRC of the 0–1 μM acetylcholine ramp.

4.7. Preserving of acetylcholine-potentiation of insulin release by an inhibitor of CK2

The M3R is regulated by phosphorylation by various kinases, including GPCR kinases and CK2 [53]. Treatment of mouse pancreatic islets with CX4945, a highly selective inhibitor of CK2, greatly reduces agonist-induced phosphorylation of β-cell M3Rs and augments M3R-stimulated insulin release in mouse and human islets [47]. Results of the present paper suggest the possibility that phosphorylation of M3Rs participates in the mechanism by which glucolipotoxicity reduces acetylcholine potentiation of insulin secretion, because a specific CK2 inhibitor protected mouse and human islets from mild and partially from severe glucolipotoxicity. However, the beneficial effect of CK2 inhibition could also be due to its direct effect on the β-granule phosphoprotein kinesin heavy chain (KHC) involved in β-granule transport along microtubules in pancreatic β-cells. It has been shown that at basal glucose concentrations and relatively low cytosolic [Ca\(^{2+}\)], KHC is phosphorylated by constitutively active CK2 resulting in low kinesin activity and β-granule transport. In contrast, at stimulatory glucose concentrations cytosolic [Ca\(^{2+}\)] is markedly increased [2,3], resulting in activation of protein phosphatase 2B and KHC dephosphorylation. As a result, kinesin ATP-dependent motor activity is activated and β-granule transport along microtubules triggered [4]. Thus, in this scenario, CK2 inhibition may prevent KHC phosphorylation and promote granule transport along microtubules.
To summarize: the present study provides a critical methodological evaluation of the pancreatic islet culture model widely used to study glucolipotoxicity and demonstrates its usefulness for studying the glucolipotoxicity phenomenon with isolated normal mouse and human islets. The present study shows for the first time glucolipotoxic impairments of cholinergic potentiation of GSIS in isolated mouse and human islets. The results of the present paper also strengthen the view that phosphorylation of β-cell M3Rs by CK2 is of pathophysiological and potential clinical relevance.

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CONFLICT OF INTEREST

None declared.

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