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MOLECULAR METABOLISM

Accumulation of 3-hydroxytetradecenoic acid: Cause or corollary of glucolipotoxic impairment of pancreatic β-cell bioenergetics?

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ABSTRACT

Objectives: Hyperglycemia and elevated blood lipids are the presumed precipitating causes of β-cell damage in T2DM as the result of a process termed "glucolipotoxicity". Here, we tested whether glucolipotoxic pathophysiology is caused by defective bioenergetics using islets in culture.
Methods: Insulin secretion, respiration, ATP generation, fatty acid (FA) metabolite profiles and gene expression were determined in isolated islets treated under glucolipotoxic culture conditions.
Results: Over time, chronic exposure of mouse islets to FAs with glucose leads to bioenergetic failure and reduced insulin secretion upon stimulation with glucose or amino acids. Islets exposed to glucolipotoxic conditions displayed biphasic changes of the oxygen consumption rate (OCR): an initial increase in baseline and Vmax of OCR after 3 days, followed by decreased baseline and glucose stimulated OCR after 5 days. These changes were associated with lower islet ATP levels, impaired glucose-induced ATP generation, a trend for reduced mitochondrial DNA content and reduced expression of mitochondrial transcription factor A (Tfam). We discovered the accumulation of carnitine esters of hydroxylated long chain FAs, in particular 3-hydroxytetradecenoyl-carnitine.
Conclusions: As long chain 3-hydroxylated FA metabolites are known to uncouple heart and brain mitochondria [53—55], we propose that under glucolipotoxic condition, unsaturated hydroxylated long-chain FAs accumulate, uncouple and ultimately inhibit β-cell respiration. This leads to the slow deterioration of mitochondrial function progressing to bioenergetics β-cell failure.

Keywords Pancreatic islets; Fatty acids; Oxygen consumption; Insulin secretion; Mitochondrial fatty acid oxidation

1. INTRODUCTION

Individuals at high genetic risk for type 2 diabetes (T2DM) that develop pancreatic β-cell dysfunction are unable to secrete sufficient amounts of insulin to compensate for insulin resistance that may arise because of age, nutritional over supply or physical inactivity, resulting in obesity and ultimately in frank hyperglycemia. Hyperlipidemia and hyperglycemia are characteristics of T2DM that are implicated as aggravating factors or actual causes of β-cell dysfunction mediated by a condition termed "glucolipotoxicity"—the combined, deleterious effect of elevated glucose and FA levels on β-cell function and survival. A consensus on the molecular cause(s) of β-cell glucolipotoxicity has not been reached despite considerable research effort. Still, various mechanisms that could explain fatty acid-induced β-cell dysfunctions have been proposed [1—7]. One of the processes implicated in fatty acid-induced pancreatic β-cell dysfunction is impairment of bioenergetics [8—11]. Faulty bioenergetics as a cause of defective insulin secretion is a plausible biochemical explanation, because the end product ATP, generated by metabolism of the physiological fuel glucose, amino acids, and probably less so FAs, serves as the obligatory coupling factor in fuel stimulated insulin release involving β-cell-specific mechanisms [12]. Basic tests of cell bioenergetics are measurements of tissue ATP content or the oxygen consumption rate (OCR). Such data on islet cells that are chronically exposed to glucolipotoxicity are limited [13—15]. Nevertheless, the literature proposes numerous ways by which FAs may alter mitochondrial energy metabolism, some discussed here. **1)** FAs may act as uncouplers and inhibitors of mitochondrial respiration [16], operating as protonophores or inhibitors of electron transport, respectively [17—19]. **2)** FAs may alter mitochondrial membrane permeability by opening the permeability transition pore [20—23]. **3)** FAs may serve as substrate for transport by the adenine nucleotide transporter (ANT), inhibiting ATP and ADP exchanges across the mitochondrial membrane [24]. **4)** FAs may act as complex-I-directed inhibitors [25]. **5)** FAs may act indirectly

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by inducing uncoupling protein (UCP2) in pancreatic islets [8–10]. Since UCP2 modulates the efficiency of ATP production [26] by catalyzing the translocation of protons across the mitochondrial membrane, one should expect changes in oxygen consumption and oxidative ATP synthesis. Previously, we reported that glucose-stimulated, but not FCCP-uncoupled, oxygen consumption is impaired in islets from T2DM organ donor [27]. The total ATP concentration in islets exposed to FA is also decreased [14,28]. Since insulin granules contain ATP, which is co-secreted with insulin, it is difficult to dissociate between the effects of FA on ATP syntheses and changes of ATP content in insulin granules or β-cell insulin store size. In fact, insulin content is decreased in islets chronically exposed to free FAs (FFAs) [14,15,28], which may result in concomitant decreased total ATP concentration and the functional ATP pool size. Recently, it has been shown that an excessive lipid supply in skeletal muscle leads to mitochondrial overload and incomplete FA oxidation, in which a large proportion of FAs entering the mitochondria are only partially degraded [29]. The latter effect may lead to accumulation of toxic long-chain acyl-CoA esters, including hydroxylated FAs, as it has been reported in genetic defects of long-chain L-3-hydroxyacyl-CoA dehydrogenase or the mitochondrial trifunctional protein [30]. The effects of fuel overload on mitochondrial FA oxidation in insulin-producing cells, to our knowledge, have not been sufficiently addressed. The goal of the present study was to evaluate the interconnection between insulin secretion and mitochondrial bioenergetics using simultaneous measurements of oxygen consumption, mitochondrial FA oxidation and hormone release in perifused isolated islets activated by fuel and non-fuel stimuli. We aimed to correlate these changes with the islet ATP content and expressions of mitochondrial genes. Since impairments of islet bioenergetics were indeed observed as a result of lipotoxic treatment, efforts were made to identify the biochemical basis of the impairment. A search was made for potentially toxic FA metabolites that might accumulate, and we identified 3-hydroxytetradecenoic acid as a potential mediator of lipotoxicity.

2. RESEARCH DESIGN AND METHODS

2.1. Animals

B6D22F1 mice (Jackson Laboratory) were used throughout. The mice were maintained on a 12:12 light–dark cycle and fed a standard 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

Mouse islets were isolated 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% 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.

2.3. Human islets

Human islets were received from the accredited Human Islet Resource Center at the University of Pennsylvania. The pancreas was procured and the isolation performed according to previously described protocols [31,32]. Altogether, we studied pancreatic islets isolated from 8 individuals (5 male and 3 female organ donors). All individuals were normoglycemic at organ isolation and ranged in age from 19 to 45 years. The time delay from the isolation to transferring the tissue to our laboratory ranged from 1 to 3 days. During this interim period, islets were kept under standard culture conditions in medium at 5 mM glucose and 25 °C [32]. The culture conditions used in our laboratory 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 [33]. For the palmitate and oleate mixture (2:1; final concentration 5 mM), 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 nominal molar FA/BSA ratio of 3.3). Since the culture medium also contained 10% FC serum, which was not defatted, the total albumin content was about 1.5%. Some uncertainty still remains about the final lipid and free fatty acid concentrations and compositions because their content in FC serum was unfortunately not determined here. Note, however, in healthy individuals, the level of FFAs has been reported to range between 0.2 and 0.7 mM, whereas in diabetics the levels are often higher and may reach concentrations of 1.0 mM [34–36].

2.5. Islet batch incubation studies

Islets were isolated as above, handpicked, 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 measurements. Pelleted islets were washed twice with cold glucose-free Hanks' buffer, and homogenized with 100 μl of 1% triton in Tris–EDTA buffer. Protein concentration was measured in islet lysates using the Bradford method.

2.6. Perifusion of islets for simultaneous measurement of oxygen consumption and insulin release

Islets (600) were loaded into the 200 μl chamber using a P200 pipette with the gel-loading tip and were allowed to settle for 1 min before resuming the flow at 80 μl/min. The perifusion apparatus consisted of a peristaltic pump, a water bath (37 °C), a gas exchanger (artificial lung: media flowed through the thin-walled silastic tubing loosely coiled in a glass jar that contained 20% O_2 and 5% CO_2 balanced with N_2), and a fraction collector (Waters Division of Millipore) [37]. In this set of experiments, Krebs buffer (pH 7.4) containing 1% of BSA (fraction V, fatty acid free) was used.

2.7. Optical method for oxygen consumption measurements

Oxygen partial pressure was measured by a phosphorescence quenching method, using a new oxygen-sensitive phosphorescent porphyrin-dendrimer Oxyphor G3 (palladium-tetrabenzoporphyrin, encapsulated inside gen 2 poly-arylglycine (AG) dendrimer) [38]. The periphery of the dendrimer is modified with oligoethyleneglycol residues, which makes the probe highly water soluble and biologically inert. In contrast to earlier generation oxyphors, oxyphor G3 does not bind to serum albumin in the perifusion medium. Thus, there are no competitive interactions between the dye and FAs for binding to albumin, which would interfere with the O_2 measurements.

The measured phosphorescence lifetime may be converted to oxygen pressure using the Stern–Volmer relationship:

$$1/\tau_0 = 1/\tau_0 + k_Q * [P_{02}]$$

where τ_0 is the phosphorescence lifetime in the absence of oxygen, τ is the lifetime at oxygen pressure P_{02}, and k_Q is a constant describing the efficiency of quenching.

The inflow oxygen tension was measured in the absence of islets in the chamber before and after each experiment. Oxygen consumption by the islets was calculated from the difference in oxygen partial pressure between the influent and the effluent (oxygen extraction) and the rate at which medium flowed through the chamber.

2.8. ATP measurements

The ATP assay was described previously [39]. Briefly, 10 μl of 5% trichloroacetic acid was added to the rapidly prepared islet pellet and maintained at room temperature for 5 min. After adding 90 μl of buffer (0.1 mM Tris acetate buffer with 1% Triton X-100, pH 8.0), the sample was then homogenized. Samples were stored at −80 °C until assay. ATP was assayed in triplicate by a luminimetric method using an ATP assay kit (Enliten ATP assay kits; Promega).

2.9. Insulin measurements

Insulin in the effluent was measured by radioimmunoassay [40,41].

2.10. Gene expression studies

Islet RNA was extracted from mouse and human islets cultured for 3 or 5 days with FAs and different glucose concentrations. The reverse transcription reaction and quantitative real time PCR (Applied Biosystems SYBR Green Master Mix kit) were used to explore the expression of selected genes critical for the metabolic and signaling pathways studied here and were performed as described previously. Data were calculated using GAPDH as an internal reference.

2.11. Mitochondrial DNA measurements

DNA was extracted from 150 to 200 islets in digestion buffer (100 mM Tris–HCl pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% w/v SDS, 1 mM β-mercaptoethanol) supplemented with 0.3 mg/mL proteinase K (Sigma) overnight at 55 °C, followed by an additional 0.3 mg/mL of proteinase K for 1 h at 55 °C. Nucleic acids were precipitated by addition of 1.25 M NaCl and ethanol, washed in 70% v/v ethanol, and resuspended in Tris–EDTA buffer supplemented with 80 μg/mL RNaseA (Roche). 15 ng of total DNA per reaction were analyzed by quantitative real-time PCR on an Applied Biosystems Step One Plus system using a standard curve method. Quantification of the mtDNA encoded gene *MT–CO1* was normalized to that of the nuclear gene *Ndufv1* using the following primer pairs, as described [42]: *MT–CO1* [(F) 5-TGC TAG CCG CAG GCA TTA C-3; (R) 5-GGG TGC CCA AAG AAT CAG AAC-3], *Ndufv1* [(F) 5-CTT CCC CAC TGG CCT CAA G-3; (R) 5-CCA AAA CCC AGT GAT CCA GC-3].

2.12. Measurements of islet acylcarnitine profile

Acylcarnitine species were measured using electrospray ionization direct injection tandem mass spectrometry. The method was adapted from an assay based upon cultured skin fibroblasts [30]. Sonicated homogenate from 700 to 800 individual islets (3–4 mice) were analyzed. For the assay, an internal standard consisting of a mixture of 8 [^{13}C]-labeled acylcarnitine species ranging from C0 (free carnitine) to C16 (palmitoyl-carnitine) were added to the sonicated islet preparation. The concentration of these internal standards was tenfold lower than in the cited fibroblast assay. Proteins present in the preparation were precipitated by the addition of 9 volumes of ethanol, followed by centrifugation and removal of the supernatant. An aliquot of the supernatant was placed into 96 well microtitre plates along with standards, controls and blanks. The supernatants were dried down under a constant stream of warm air or by heating for 15 min at 65 °C, when the butyl-derivatives were made by addition of butanolic HCL, and evaporated to dryness under a stream of air. Final reconstitution was made by the addition of acetonitrile:water (1:1), the volume of which was then adjusted to the required sensitivity. This product was then directly injected into a Waters Xevo TQS tandem mass spectrometer. Data were analyzed in the multiple reaction monitoring (MRM) mode with data gathered for the parent ions of those compounds with a characteristic fragment of m/z 85, which is unique for the carnitine moiety.

2.13. Statistical analysis

Data are presented as the mean ± SE of three to four experiments. In appropriate cases, significant differences between groups were determined by ANOVA with post hoc analysis using Dunnett's multiple comparison test. $p \leq 0.05$ was considered significant.

3. RESULTS

In order to examine the effects of FAs on bioenergetics in pancreatic islet cells using a tissue culture system, various analytical approaches were employed: ATP measurements, studies of oxygen consumption, determination of mRNA expressions of mitochondrial genes and of the fatty acid acylcarnitine profiles.

3.1. Changes in islet ATP concentrations

ATP concentrations were measured in islets immediately at harvest and also in islets cultured for 3 days under glucolipotoxic conditions but then subjected to 60 min preincubation in a physiological buffer without glucose or FAs present and then stimulated with different glucose concentrations (3 and 10 mM) (Table 1). Islet ATP content at harvest after a 3-day culture with a mixture of palmitic acid (PA) and oleic acid (OA) (0.5 mM; 2:1 ratio) and 10 or 16 mM glucose, was

Table 1 – Islet ATP content and glucose-induced changes in ATP concentration (pmole/islet).

3 day culture conditions	ATP content at harvest	ATP content after 60 min incubation with low or high glucose	
		at 3 mM	at 10 mM
G, 10 mM	13.37 ± 0.75	7.75 ± 0.65	10.40 ± 1.4*
G, 10 mM + PA + OA	10.14 ± 0.86#	5.85 ± 0.05#	6.79 ± 1.24
G, 16 mM	12.00 ± 1.60	7.51 ± 0.3	9.34 ± 1.20*
G, 16 mM + PA + OA	10.26 ± 1.69	5.48 ± 0.8#	6.28 ± 0.28

ATP content was measured by harvesting 10 islets at the end of 3 days of culture in RPMI 1640 medium containing 10 or 16 mM glucose ± a mixture of PA and OA (0.5 mM; 2:1 ratio). After 3 days culture other sets of islets (10 islets per group) were preincubated with glucose-free Krebs–Ringer bicarbonate buffer with 0.25% BSA for 60 min to achieve fuel depletion. Islets were then incubated with two different glucose concentrations (3 and 10 mM) for another 60 min to assess the capacity to recover. Islets were then collected and ATP concentration was measured as described previously [30].

Results are presented as means ± SE of 4 experiments. The ATP contents are given in units of pmole/islet. #- $p \leq 0.05$ compared with ATP content with or without FA at the same glucose level (harvested after culture or after batch incubation); *- $p \leq 0.05$ compared to baseline of 3 mM glucose (in case of batch incubation).

reduced by 32 and 17%, respectively, when compared to culture condition with 10 or 16 mM glucose alone. The moderately reduced ATP content of FA treated islets is probably the result of the markedly reduced insulin pool (as low as 50%) as shown in comparable previous reports [14,15,28]. When mouse islets pretreated without fuel present were allowed to recover for 60 min at 3 mM glucose, the ATP concentration was relatively low in both groups of islets when compared to the ATP content in islets harvested right after culture. The ATP concentrations at the 3 mM glucose baseline in islets previously exposed in culture to FA and 10 or 16 mM glucose were 33 and 37% lower, when compared to islets cultured with 10 or 16 mM glucose alone, respectively. Increasing the glucose concentration from 3 to 10 mM in the test system raised ATP by 34 and 24% in control islets cultured with 10 or 16 mM glucose alone, respectively. When islets had been cultured with a mixture of PA and OA, 10 mM glucose in the test system did not significantly increase the islet ATP concentration. Collectively, these results suggest that islets cultured under glucolipotoxic conditions suffer a marked perturbation of energy metabolism. This is illustrated most compellingly by the limited capacity to reenergize after fuel depletion when islets were incubated for 1 h with 10 mM glucose (ideal for culturing rodent islets). Several explanations for these results are possible, including augmented ATP consumption or impaired oxidative phosphorylation as obvious possibilities.

3.2. Islet oxygen consumption following culture under glucolipotoxic conditions

Based on ATP measurements, we expected that 3-day exposure of mouse isles to FAs and high glucose would lead to impaired mitochondrial function manifested by a decreased oxygen consumption rate (OCR). Figure 1 presents the simultaneous measurements of the OCR and of insulin secretion. We found that the baseline of OCR was greatly elevated in islets cultured for 3 days with 16 mM glucose and 0.5 mM PA compared to islets cultured at 10 or 16 mM glucose alone (Figure 1A). In addition, step-wise increases of glucose concentrations led to high OCR in all islets groups, reaching saturation at 12 mM glucose. Importantly, the V_{max} of OCR was higher in PA-treated islets and the glucose dependency curve was unchanged. An amino acid mixture (of 19 amino acids at physiological levels of 4 mM [43]) changed the OCR slightly but only in the PA-treated islets, and FCCP caused similar relative changes of OCR in all groups of islets. (Note: FCCP was used without prior oligomycin treatment, because, in isolated perfused islets, it takes a prohibitively long time to achieve the full effect of oligomycin.) These changes in OCR were paralleled by a marked inhibition of glucose-stimulated insulin secretion (Figure 1B). In contrast to the lack of an effect on OCR, amino acids greatly enhanced glucose stimulated insulin secretion in controls but this effect was drastically reduced in FA treated islets. FCCP stopped hormone secretion abruptly. When islets were cultured at 25 mM glucose, the baseline of OCR was increased equally in control and PA-treated islets resulting in overlapping of OCR tracings in both groups of islets (Figure 1C). These enhancements of baseline and stimulated respiration are in marked contrast to near total loss of glucose stimulated insulin secretion in PA-treated islets and a diminished response in islets treated with glucose only (Figure 1D).

The baseline of OCR was even higher when islets were cultured for 3 days with a mixture of PA/OA at 16 mM glucose compared to culture with PA at 16 mM glucose (Figure 2A). At the same time, insulin release was greatly reduced with high glucose or an amino acids mixture as stimulus (Figure 2B).

Based on the results of ATP measurements (Table 1), one could hypothesize that the higher baseline of oxygen consumption might be due to a partial uncoupling of oxidative phosphorylation, even though the FCCP effect was similar in both groups of islets. Because uncoupling of respiration and phosphorylation may lead to time dependent general deterioration of mitochondrial function, islets were cultured for a longer duration of time (5 days) with a mixture of PA/OA and 16 mM glucose. We found that the baseline rate of oxygen consumption was reversed to a lower level than seen in islets cultured with 16 mM glucose alone (Figure 2C). In addition, stimulation of respiration by 6 and 12 mM glucose was significantly reduced. The FCCP effect was similar in both groups of islets. The reduction of the respiratory response was associated with more dramatic reduction in insulin secretion compared to islets cultured with FA for 3 days only (Figure 2D). In these studies OA, when used in a mixture with PA, did not protect islets from "glucolipotoxicity".

When changes in OCR are compared between islets cultured at standard conditions (10 mM glucose) and islets cultured for 5 days at 16 mM glucose plus a mixture of PA/OA, these data mirror, in a truly remarkable way, the previously reported OCR changes in human islets isolated from normal as compared to T2DM donors [27]. It is also worthwhile to recall that this defect of T2DM islets was rescued by activation of the glucokinase glucose sensor (using Piragliatin^{R}), thereby repairing metabolism and function.

Glucose dependency of OCR is sigmoidal and clearly shows a higher baseline and increased Vmax in islets cultured for 3 days with high glucose and the PA/OA mixture (Figure 3A). In contrast to the OCR, insulin release is decreased at higher glucose concentrations (>6 mM) (Figure 3B). However, after 5 days at the same conditions, the baseline of OCR is decreased, and its glucose dependency is right shifted and the Vmax decreased (Figure 3A). Insulin secretion is totally blocked (Figure 3B). Figure 4 presents the relationship between glucose stimulated insulin release and the OCR in analogy to the energy production/insulin secretion diagram used in a previous publication [27]. Increases in OCR led to increases in insulin release in islets cultured at 16 mM glucose with threshold between 0.3 and 0.4 nmole of oxygen per min per 100 islets (Figure 4A). Islet treatment with PA caused a higher baseline, right shift and lower V_{max} of OCR/insulin secretion curve. When the glucose concentration in the culture was increased to 25 mM, the OCR/insulin release curve shifted to the right (Figure 4B). Islet exposure to PA significantly flattened the OCR/insulin release relationship (Figure 4B). The OCR/insulin curve was also shifted to the right with lower V_{max} when islets were cultured for 3 day with a mixture of PA/OA (Figure 4C). Islets cultured for 5 days with the PA/OA mixture and 16 mM glucose completely flattened the OCR/insulin release curve (Figure 4D). The V_{max} of insulin release was also reduced in islets cultured for 5 days with 16 mM glucose alone (Figure 4D).

3.3. Mitochondrial DNA content and expression of mitochondrial genes

To better understand the changes in the OCR and ATP profiles, we measured mitochondrial DNA and the expression of genes involved in mitochondrial function. Mitochondrial DNA did not significantly change after 3-day culture with 0.5 mM PA and different glucose concentrations, although we observed a trend of decreasing mitochondrial DNA content that should not be overlooked (Figure 5A). These changes correlated with a trend of decreasing expression of transcription factor A mitochondrial (Tfam) (Figure 5B), which regulates mitochondrial gene transcription/DNA replication [44].

In mouse islets, the expression of the transcriptional co-activator peroxisome proliferator-activated receptor-γ co-activator-1 (PGC-1α), carnitine palmitoyltransferase 1A (CPT-1A), very long-chain acyl-coenzyme A dehydrogenase (Acadvl), long-chain acyl-Coenzyme A

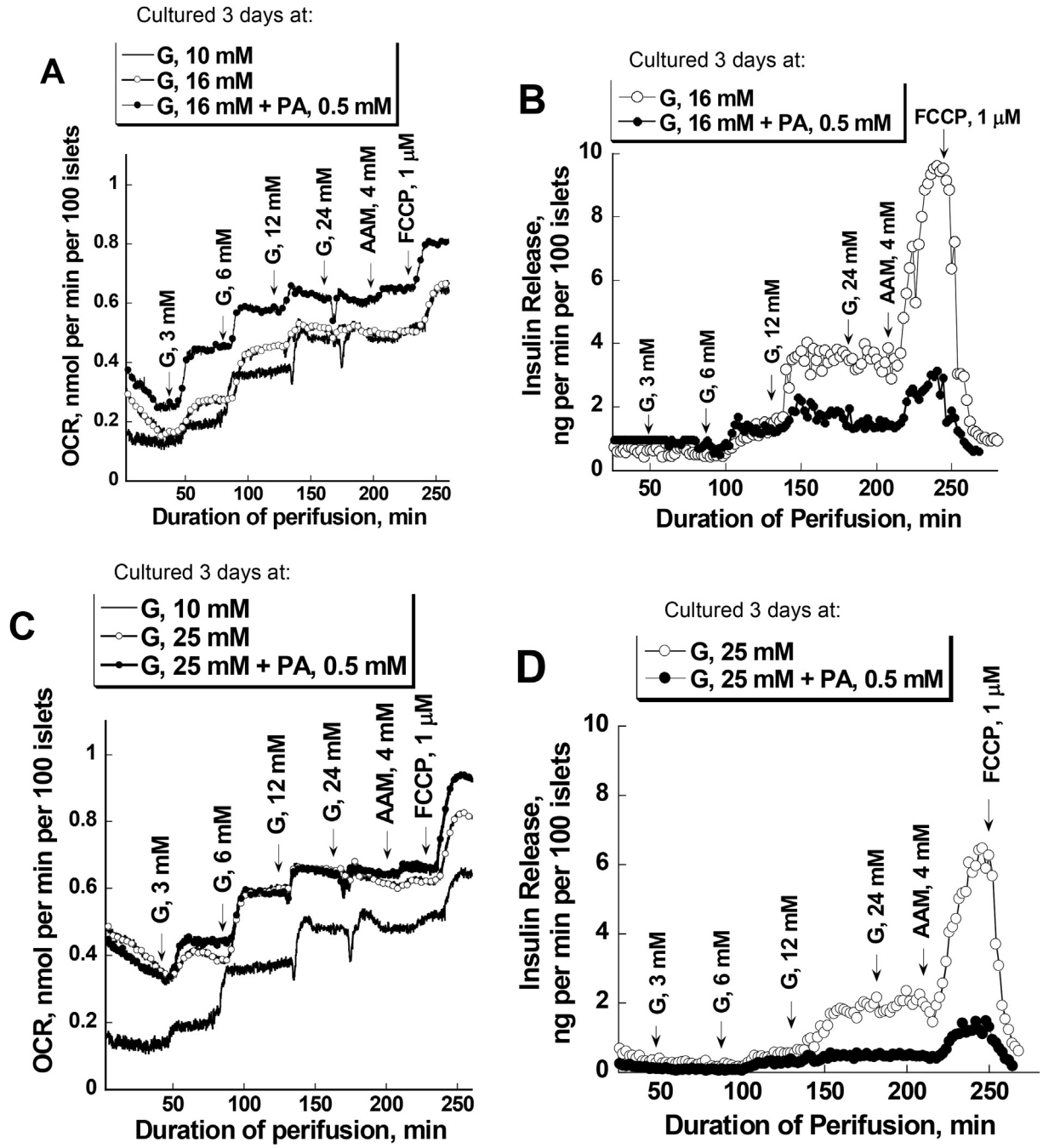

Figure 1: Effects of 3 day culture with PA and high glucose on the oxygen consumption rate (OCR) and insulin release of mouse islets. Islets were stimulated by step increases of glucose concentration (at 3, 6, 12, 24 mM) and then with a 4 mM of amino acid mixture [43] at high 24 mM glucose. Afterwards, FCCP 1 μM was added to uncouple respiration and to estimate the maximal OCR. **Panel A**: OCR of mouse islets cultured for 3 days at 16 mM glucose ± 0.5 mM PA. The OCR tracing of islets cultured at 10 mM glucose routinely used in the culture system is also presented for comparison (*clear line*: cultured at 10 mM glucose alone; *open circles*: cultured at 16 mM glucose alone; *filled circles*: 16 mM glucose plus PA). **Panel B**: Insulin release for the experiments presented in Panel A; (*open circles*: cultured with glucose alone; *filled circles*: glucose plus PA); **Panel C**: OCR of islets cultured at 25 mM glucose ± 0.5 mM PA for 3 days. OCR of islets cultured at 10 mM glucose is also presented. **Panel D**: Insulin release for the experiments presented in Panel C (except that at 10 mM glucose); (*open circles*: cultured with glucose alone; *filled circles*: glucose plus PA). Data presented as means of 3 experiments. SE omitted because the results of different traces were very close.

dehydrogenase (Acadl) and uncoupling protein 2 M (UCP2-M) were increased after a 3-day culture with 16 mM glucose and a 0.5 mM mixture of PA/OA (Figure 6A). In human islets, the expression of PGC-1α correlated with increased mRNA of cytochrome c, cytochrome c oxidase subunit 5b (Cox5b), CPT-1A, Acadvl and UCP2-M, due to culture with 16 mM glucose and 0.5 mM PA Figure 6B). In addition, Hyou1, SREBP-1C, SREP2, SERCA2 and CHOP-M were elevated (Figure 6B). It is of interest that OA (when used in mixture with PA) prevented an increase in expression of Hyou1, SREBP-1C, SREP2, SERCA2 and decreased changes in UCP2-M when compared to human

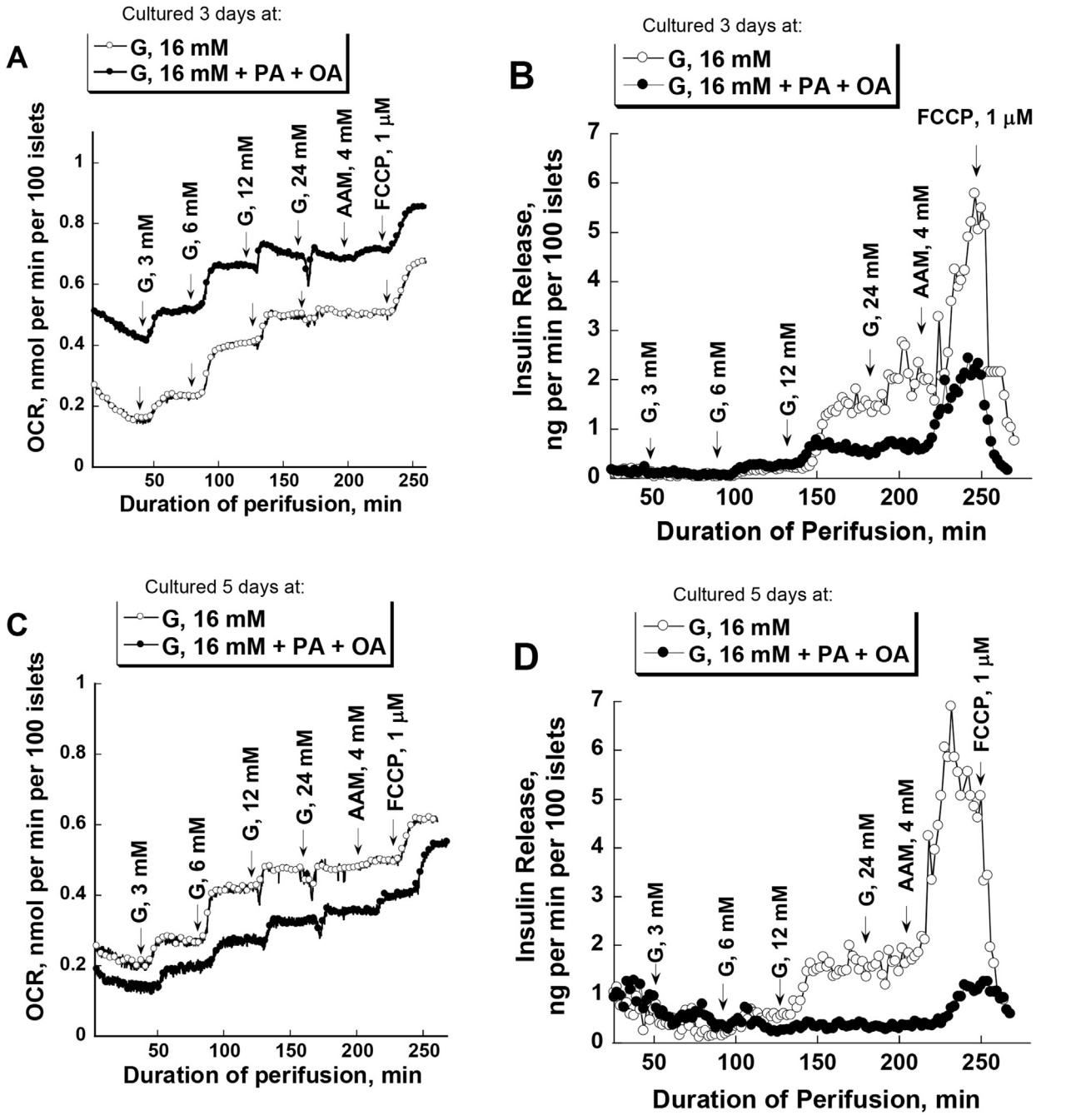

Figure 2: Oxygen consumption rate and insulin secretion in mouse islets exposed for 3 days (panels A and B) or 5 days (panels C and D) to a mixture of PA and OA. Panel A and B: OCR and insulin secretion after islets were cultured for 3 days with a mixture of PA and OA at a 2:1 ratio (total concentration was nominally 0.5 mM bound to 1% BSA); **Panel C and D**: OCR and insulin secretion after islets were cultured with the mixture of PA and OA for 5 days. Open circles: OCR and insulin release in islets cultured with glucose alone; filled circles: OCR and insulin release in islets cultured with glucose plus a PA/OA mixture. Data presented as means of 3 experiments. SE are again not shown because of the close overlap of the results.

islets cultured only with PA (Figure 6C). This apparently protective effect of OA contrasts with our unpublished observation that OA augments lipotoxicity assessed by insulin secretion data. It is important to note here that expression of PGC-1α in response to exposure to PA or the mixture of PA/OA in mouse and human islets was strongly dependent on the glucose concentration in the culture medium. mRNA of PGC-1α clearly increased at 16 mM but not at 25 mM glucose (Figure 6D–F).

3.4. Acylcarnitine profile of mouse islets acutely or chronically exposed to PA

To gain insight into β-oxidation of FAs as influenced by glucose and in the context of this investigation searching for metabolites that might cause the mitochondrial damage, we measured the acylcarnitine profile of mouse islets using tandem mass spectroscopy. Two different experimental conditions were employed before performing the measurements: 1) isolated islets were cultured at 10 mM glucose for 3

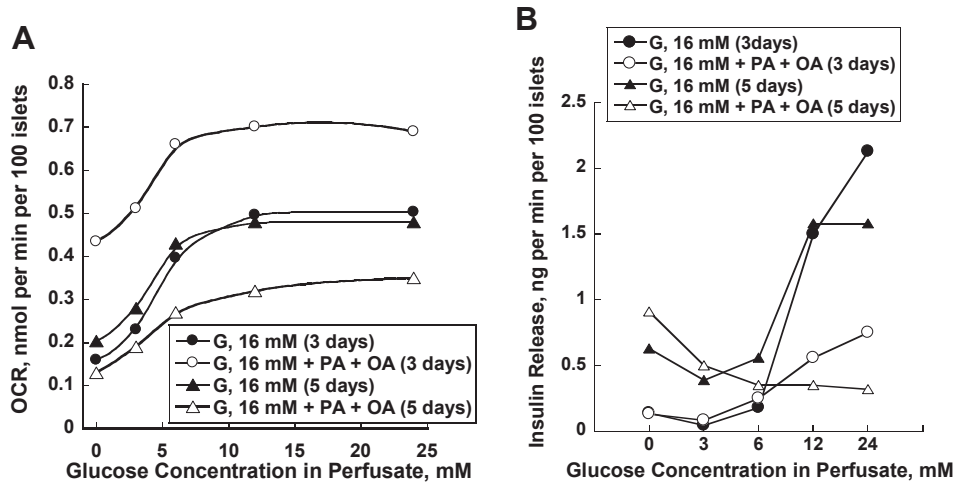

Figure 3: Panel A: Oxygen consumption rate in mouse islets cultured for 3 or 5 days at 16 mM glucose ± PA/OA as function of the glucose staircase. **Panel B**: Insulin release (for the experiments presented in panel A) as function of a glucose staircase. Each curve represents the mean of 3 experiments.

days (i.e. routine culture condition), then were subjected to batch incubation for 2 h with 0.5 mM [U-^{13}C]PA at different glucose concentrations (3 and 16 mM) and were then analyzed; 2) islets were cultured for 3 days at 0.5 mM [U-^{13}C]PA, and different glucose concentrations (5, 16 and 25 mM) and were then analyzed.

High glucose caused a significant decrease in labeled acetylcarnitine levels in both experimental conditions (Table 2). It is remarkable that the decline of the two carbon metabolite was associated with accumulation of one particular long-chain 3-hydroxy fatty acid (LC3HFA), i.e. 3-hydroxytetradecenoic acid, in both experimental conditions. Since the production of acetylcarnitine was decreased at the same time, this suggests incomplete FA oxidation. The levels of these metabolites were significantly higher when islets were cultured with 0.5 mM [U-^{13}C]PA and different glucose concentration for 3 days compared to 2 h incubation, but the overall outcome was the same, indicating that the disturbance of FA metabolism was long lasting. It should be noted that in these experiments all groups of islets were incubated or cultured with 0.5 mM [U-^{13}C]PA in order to follow the flux through specific steps of FA oxidation. Islets incubated or cultured at low glucose and 0.5 mM [U-^{13}C]PA served as controls in these experiments.

4. DISCUSSION

4.1. Role of altered bioenergetics in defective insulin secretion due to glucolipotoxicity

To our knowledge, this is the only study where dynamics of oxygen consumption and insulin secretion were simultaneously recorded in isolated rodent pancreatic islets chronically exposed to high glucose and FAs in an organ culture system. The results showed time dependent biphasic changes in oxygen consumption during chronic exposure to FAs. We observed an increased baseline of OCR with normal glucose induced enhancement of respiration in islets cultured for 3-days followed by decreased baseline as well as glucose-stimulated OCR after 5 days of islet exposure to a FAs and high glucose. These changes in OCR were associated with lower islet ATP content and a decreased glucose-stimulated rise of ATP levels and paralleled by an increased expression of UCP2 in mouse and human pancreatic islets exposed to FAs for 3 or 5 days. Surprisingly, our results also showed that the relative FCCP stimulation of oxygen consumption was similar in both groups of islets, i.e. they failed to parallel the dramatic change of Vmax with time. It has already been shown by others [8–10] that lipotoxicity induces UCP2 in pancreatic islets [8–10] and that islets lacking UCP2 are resistant to the toxic effects of PA [10]. It is important to realize that in these previous studies, the authors assumed that UCP2 operates as classical uncoupler of oxidative phosphorylation (translocators specifically of protons across the mitochondrial membrane) thereby collapsing the proton gradient [26] and leading to decreased efficiency of ATP production in pancreatic β-cells. However, views on UCP2 biochemistry have greatly changed since the studies were done. Operationally speaking, UCP2 has only mild uncoupling activity [45,46]. UCP2 catalyzes an exchange of mitochondrial malate, oxaloacetate, aspartate for cytosolic phosphate, and protons. Furthermore, it plays a unique regulatory role in cell bioenergetics by metabolic reprogramming [47]. This argues against UCP2 having typical uncoupling activity in vivo [48,49] and suggests that its central role is in reprogramming metabolic pathways [47,50]. Instead of uncoupling of oxidative phosphorylation we propose here that the higher baseline of OCR and the ATP deficit at 3 days is due to activation of ATP consuming processes leading to excessive ATP hydrolysis. Enhanced glycerolipid/FAs cycle [1,2,51], an energy demanding process, could be one of those processes. This cycle is activated in obese animals (in the adaptive state) but decreased in the diabetic state [2]. Indeed, we have shown previously that islets from T2DM donors have lower glucose-stimulated OCR with normal FCCP responses [27]. With extended duration of the glucolipotoxic condition mitochondrial function deteriorates as manifested in reduced basal OCR and abnormal energy production/insulin secretion diagrams (as summarized in Figure 4).

4.2. Long-chain 3-hydroxy fatty acids as possible cause for the reduction of oxygen consumption and oxidative phosphorylation in pancreatic β-cells

The acylcarnitine profile provides a snapshot of substrate flux through specific steps of FA catabolism. More than 25 acylcarnitine species, ranging in size from 2 to 22 carbons, were detected and quantitated in islets extracts. These acylcarnitine esters are formed from their respective acyl-CoAs by a family of carnitine acyltransferases that reside in subcellular organelles (primarily mitochondria), catalyzing the exchange of CoA for carnitine [52]. It is worth noting that these

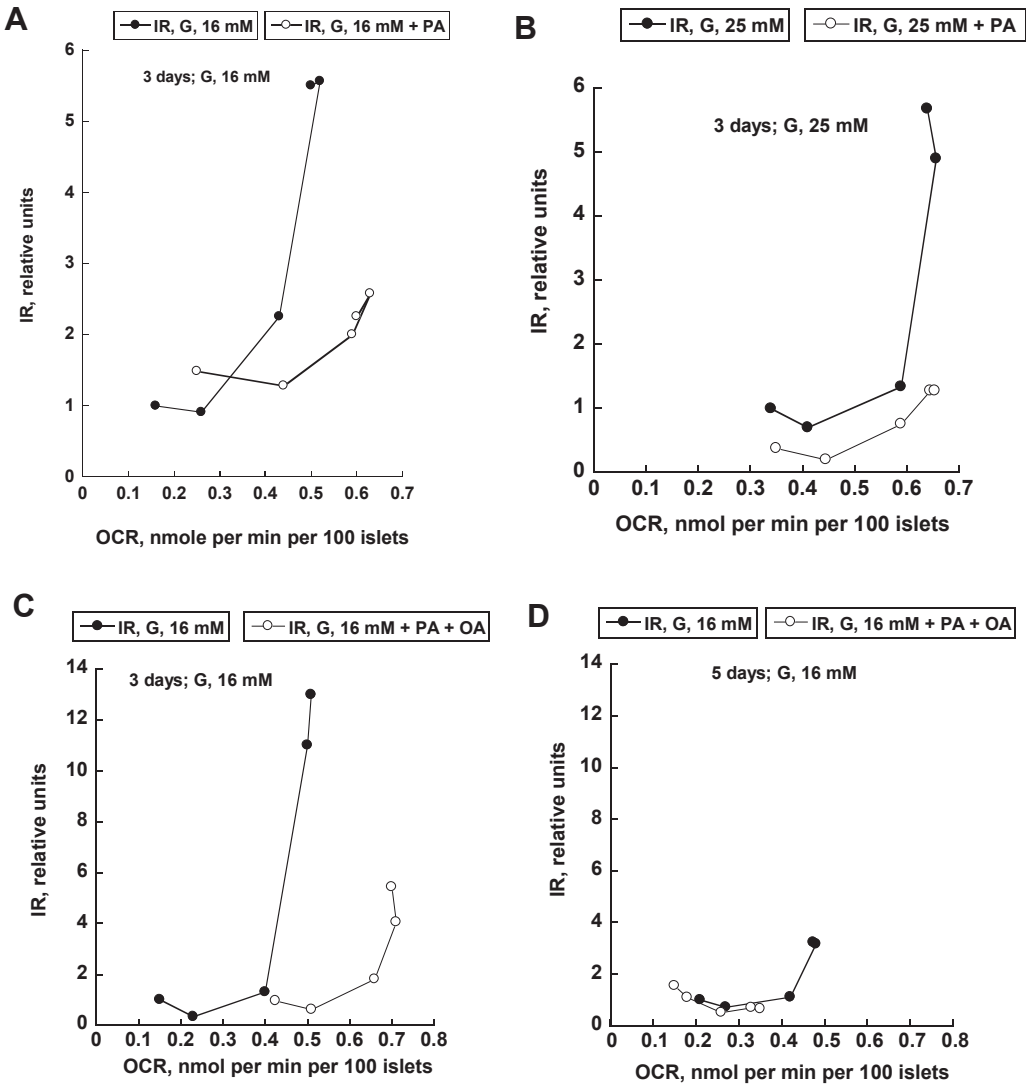

Figure 4: Relationship between insulin release and oxygen consumption rate. Panel A: mouse islets were cultured for 3 days at 16 mM glucose ± 0.5 mM PA. **Panel B**: islets were cultured at 25 mM glucose ± 0.5 mM PA for 3 days. **Panel C**: islets were cultured for 3 days with a mixture of PA and OA at the ratio 2:1 (total concentration was 0.5 mM bound to 1% BSA); **Panel D**: islets were cultured with the mixture of PA and OA for 5 days. *Filled circles*: islets cultured with glucose alone; *open circles*: islets cultured with glucose plus FA(s). Data presented as means of 3 experiments.

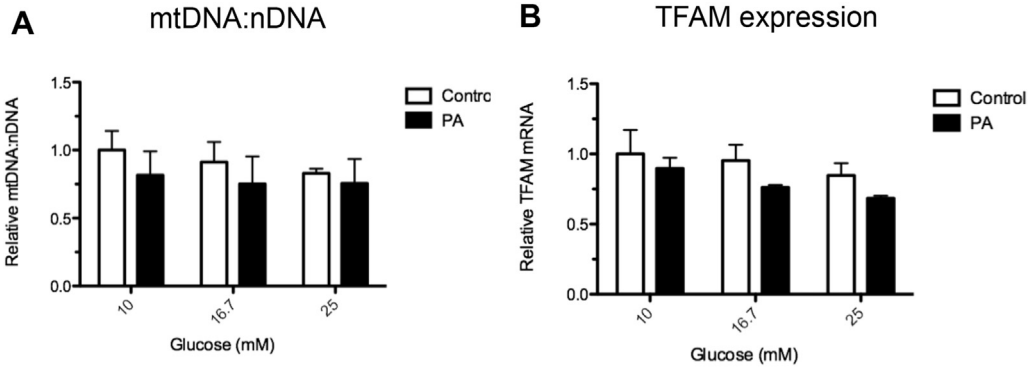

Figure 5: Mitochondrial DNA and TFAM expression in mouse islets after chronic exposure to PA and different glucose concentrations. Panel A: The relative mitochondrial DNA copy number was measured by determining the ratio of the mtDNA-encoded gene MT—CO1 to the nuclear gene NDUFV1. **Panel B**: Expression of TFAM after chronic exposure to PA at different concentrations of glucose (10, 16.7 and 25 mM). Data presented as means ± SE of 4—5 experiments.

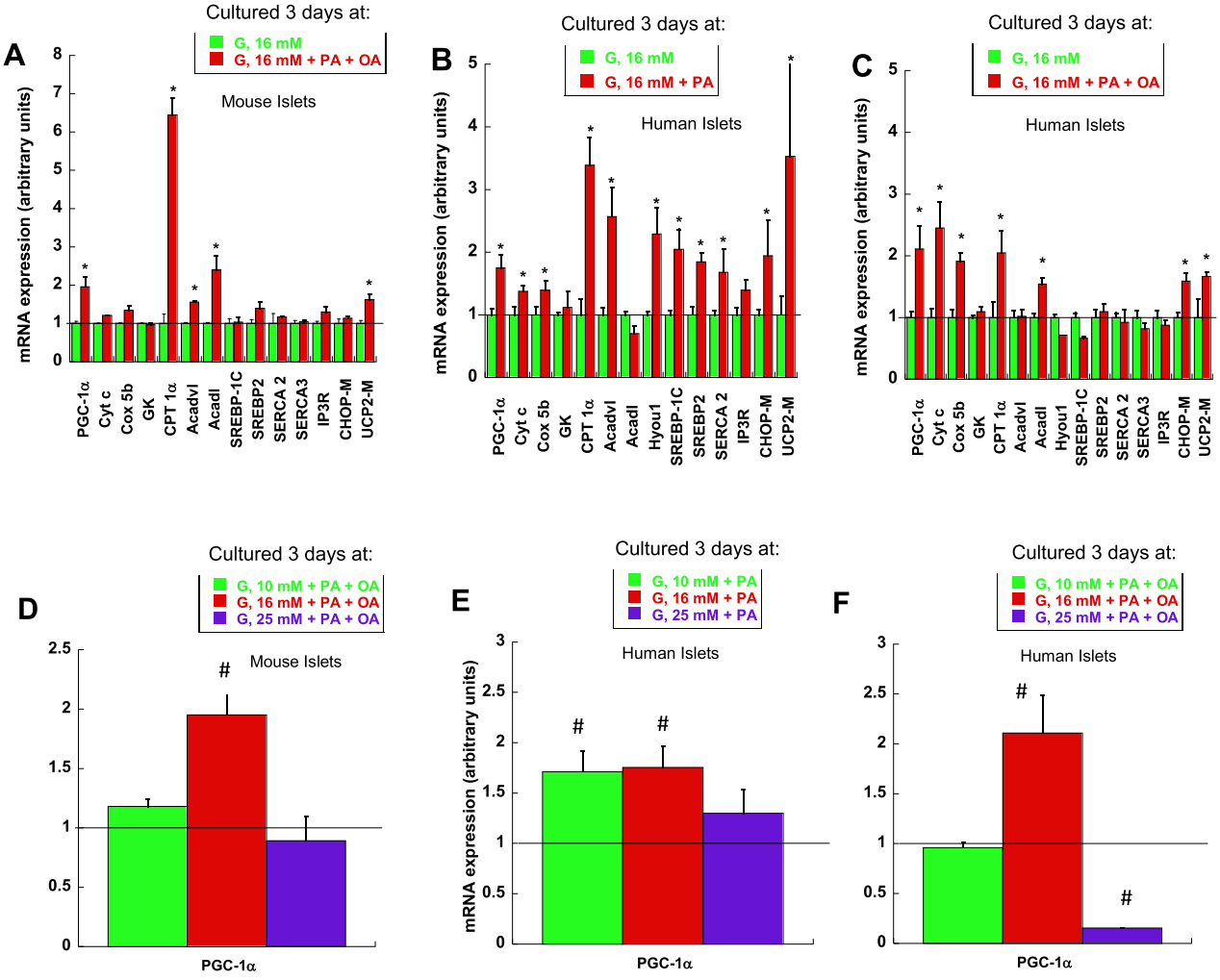

Figure 6: Gene expression profile of mouse and human islets exposed to FAs. Panel A: Gene expression profile of mouse islets exposed for 3 days to 16 mM glucose and a mixture of PA and OA. **Panel B and C**: Gene expression profile of human islets exposed for 3 day to 16 mM glucose and PA (B) or a mixture of PA and OA (C). Data were normalized to the expression in islets cultured at 16 mM glucose alone (*- $p \leq 0.05$ compared to 16 mM glucose alone). **Panel D—F**: Glucose dependence of PGC-1α expression in mouse (D) and human islet (E and F) due to 3 day exposure to PA or PA/OA mixture. PGC-1α expressions in islets cultured at different glucose level and PA/OA mixture were compared to the expression at the same glucose levels alone which were normalized to 1 (#- $p \leq 0.05$ compared to same glucose alone). Data presented as means ± SE of 4—5 experiments.

carnitine acyltransferases catalyze equilibrium reactions allowing the extrapolation that the corresponding CoA esters change in parallel. This strategy of measuring acyl-carnitine profiles as indicators of biochemical mechanisms has been used previously to study regulation of metabolism of skeletal muscle and cultured myotubes [29]. It was discovered that β-oxidation of FAs in related disease models was incomplete and associated with the accumulation of various FA metabolites caused by lipid oversupply, apparently resulting in insulin resistance. Results of the present study similarly show that acute or chronic glucolipotoxicity conditions lead to selective accumulation of the carnitine esters 3-hydroxytetradecenoic acid, an intermediate of oleic acid metabolism (3OH-U-13C14:1). Quantitatively, the accumulation is astonishing. This accumulation was associated with decreased acetyl-carnitine, the end product of β-oxidation, suggesting incomplete FA oxidation. It is known that LC-3OH-FAs (specifically the unsaturated 14:1) accumulate in genetic deficiencies of the mitochondrial trifunctional protein (MTP), comprising the activities of long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), long-chain 2-enoyl-CoA hydratase and long chain 3-oxoacyl-CoA thiolase (LKAT) (47, 48). Mutations of MTP-encoding genes cause isolated enzyme deficiencies or a deficiency of all three components of MTP. These diseases are biochemically characterized by tissue accumulation and urinary excretion of multiple long-chain 3-hydroxyacyl carnitine esters including 3OH-C14:1. In contrast, acylcarnitine profiles in flavin dependent, very long-chain acyl-CoA dehydrogenase reveal high levels of the strait-chain C14 acylcarnitines with some hydroxylated C16 species. It was also reported that LC3HFAs induce oxidative stress and disrupt mitochondrial homeostasis in rat brain [53,54] and behave as strong uncouplers/inhibitors of oxidative phosphorylation in rat heart mitochondria [55] at concentrations that might occur in islet tissue. It should be stressed here that the mechanisms underlying the uncoupling effects of hydroxylated acyl-CoA esters are different from those provoked by non-hydroxylated long-chain FAs [14,56]. The mechanism by which FAs are generally thought to uncouple the oxidative phosphorylation involves mitochondrial inner membrane anion carriers such as adenine nucleotide translocator, the inhibitor of which, atractyloside, abolished the uncoupling effect induced by palmitic acid in skeletal muscle mitochondria [57]. Atractyloside does not prevent the

Table 2 — ^{13}C-acylcarnitine profiles of mouse pancreatic islets at control and glucolipotoxic conditions.

Duration of exposure	Acute effects (2 h incubation)		Chronic effects (3 days in culture)		
Acyl-carnitine esters	*Batch Incubation Condition* G, 3 mM + [U-13C]PA, 0.5 mM	G, 16 mM + [U-13C]PA, 0.5 mM	*Culture Conditions* G, 5 mM + [U-13C]PA, 0.5 mM	G, 16 mM + [U-13C]PA, 0.5 mM	G, 25 mM + [U-13C]PA, 0.5 mM
Palmitoyl-carnitine (^{13}C-C16)	**4.25 ± 0.93**	**5.64 ± 2.71**	**79.63 ± 43.11**	**33.23 ± 15.03**	**33.13 ± 17.02**
Hexadecenoyl (^{13}C-C16:1)	1.29 ± 0.47	2.08 ± 0.89	3.70 ± 1.15	3.60 ± 1.05	4.13 ± 1.29
^{13}C-3-OH-C16	0.41 ± 0.15	0.50 ± 0.21	2.60 ± 0.20	2.367 ± 0.32	2.76 ± 0.58
^{13}C-3-OH-C14	0.97 ± 0.29	1.84 ± 0.55	9.00 ± 2.21	10.60 ± 1.15	12.37 ± 0.79
Tetradecenoyl (^{13}C-C14:1)	0.30 ± 0.20	0.67 ± 0.47	5.20 ± 2.21	4.66 ± 1.97	5.07 ± 1.80
3-hydroxytetradecenoyl-carnitine (^{13}C13-3-OH-C14:1)	**6.12 ± 1.09**	**20.36 ± 3.70***	**71.60 ± 4.25**	**151.2 ± 6.57***	**202.7 ± 28.9***
Tetradecadienoyl (^{13}C-C14:2)/Suberyl (^{13}C-C6DC)	1.43 ± 0.46	1.92 ± 0.62	8.36 ± 5.37	11.40 ± 7.9	7.83 ± 4.23
Dodecanoyl-carnitine (^{13}C-C12)	0.36 ± 0.13	0.48 ± 0.30	1.80 ± 0.45	1.97 ± 0.49	1.73 ± 0.120
Dodecenoyl-carnitine (^{13}C-C12:1)	0.43 ± 0.21	0.59 ± 0.40	3.60 ± 1.48	2.06 ± 0.37	3.17 ± 0.13
Dodecadienoyl (^{13}C-C12:2)/(^{13}C-C6-DC)	2.04 ± 0.64	3.42 ± 1.36	10.30 ± 2.91	9.40 ± 2.08	12.83 ± 4.25
Decanoyl-carnitine (^{13}C-C10)	0.33 ± 0.16	0.39 ± 0.14	2.10 ± 0.173	2.17 ± 0.50	1.93 ± 0.08
Cis-4-Decenoyl (^{13}C-C10:1)	0.34 ± 0.06	0.43 ± 0.09	1.30 ± 0.10	1.17 ± 0.28	1.53 ± 0.20
Decadienoyl (^{13}C-C10:2)/Methylmalonyl	0.75 ± 0.21	1.17 ± 0.31	4.80 ± 0.69	3.87 ± 0.54	3.63 ± 1.01
Octanoyl-carnitine (^{13}C-C8)	0.56 ± 0.14	0.74 ± 0.25	3.30 ± 0.45	3.07 ± 0.12	2.93 ± 0.52
Octenoyl-carnitine (^{13}C-C8:1)	0.82 ± 0.39	1.18 ± 0.76	6.10 ± 0.43	4.93 ± 0.684	4.96 ± 0.63
Hexanoyl-carnitine (^{13}C-C6)	0.73 ± 0.26	1.26 ± 0.46	20.63 ± 15.4	15.83 ± 10.7	11.17 ± 6.37
Butyryl/isobutyryl (^{13}C-C4)	1.13 ± 0.31	1.63 ± 0.65	9.87 ± 4.09	12.77 ± 4.38	10.63 ± 2.83
3-OH-Butyryl-carnitine (^{13}C-C4-OH)	0.83 ± 0.29	1.63 ± 0.53	9.60 ± 2.17	9.53 ± 0.29	9.37 ± 1.58
Acetyl-carnitine (^{13}C-C2)	**45.91 ± 3.2**	**28.16 ± 6.3***	**188.25 ± 3.75***	**98.25 ± 5.85***	**100.35 ± 15.75***

Two experimental conditions were used: 1) islets were cultured at 10 mM glucose for 3 days (i.e. routine culture condition) and were then subjected to batch incubation for 2 h with 0.5 mM [U-^{13}C]PA and different glucose concentrations: 3 and 16 mM (n = 3); 2) islets were cultured for 3 days at 0.5 mM [U-^{13}C]PA and different glucose concentrations: 5, 16 and 25 mM. Islets were harvested after batch incubation (1) or after culture (2) and acylcarnitine profiles analyzed by tandem mass spectroscopy (n = 3); Critical intermediates of FA metabolism are highlighted. The acyl-carnitine ester concentrations are given in units of nmole/mg protein. *-p ≤ 0.05 compared to baseline at 3 or 5 mM glucose.

effects of hydroxylated long-chain FAs on mitochondrial oxidative phosphorylation, but the exact mechanism of action of these unique metabolites has not been fully identified.

It is remarkable that the concentration of 3-hydroxytetradecenoyl acid carnitine ester in mouse islets after a 3-day culture with high glucose and PA increased 2.3 times compared to 2 h incubation, indicating a relatively slow time course for reaching an equilibrium. Based on these data, we project that if glucolipotoxic conditions are extended to 5 days, this effect may be even stronger. It is realized that the uncoupling/inhibition efficiency of LC3HFA is almost certainly stronger than that of the CoA or carnitine esters of the acid. The possible accumulation of free LC3HFAs and their effects on oxidative phosphorylation in pancreatic β-cells has not been studied, and it remains to be seen whether and at what level the free LC-3OH-FAs accumulate in islet tissue. However, as already indicated, acylcarnitine transferases are equilibrium enzymes, which implies that the acyl-CoA ester profile would change accordingly in glucolipotoxic conditions. Furthermore, tissues probably contain acyl-CoA hydrolases, which might facilitate the accumulation of free acids. Further experiments are needed with isolated β-cell mitochondria using concentrations of free LC3HFAs that are found in tissues under glucolipotoxic conditions.

Based on the literature and our data, we propose a novel mechanism of glucolipotoxicity (Figure 7). High glucose and FAs lead to activation of the energy wasting Glycerolipid/FA cycle [1,2] reflected in increased OCR. At the same time, glucose increases the mitochondrial redox state through an unbridled push mechanism due to the operation of glucokinase [58], resulting in inhibition of LCHAD in the MTP and crotonase branches of FA oxidation. In addition, at high levels of palmitoyl CoA/oleoyl-CoA, the pathway is overloaded, its flux appears to drop, and metabolites accumulate due to competition between acyl CoAs of different chain lengths for a set of acyl-CoA dehydrogenases with overlapping substrate specificity [29]. All of these processes result in the accumulation of acyl-CoA-esters, primarily 3-hydroxytetradecenoic acid (3-OH-C14:1) in the case of islet tissue. The free acids formed by acyl-CoA hydrolase are hypothesized to inhibit oxidative phosphorylation. Time-dependent deterioration of mitochondrial function and a decrease in mitochondrial and cellular mass would be the consequence. We thus propose that FA-mediated bioenergetic failure is the main cause of glucolipotoxicity, resulting in impaired nutrient-stimulated insulin secretion, and associated with impaired neuro-entero-endocrine potentiation of insulin release as illustrated by defects of acetylcholine and GLP-1 potentiation of glucose stimulated insulin secretion [33,59–61].

4.3. Critical perspective

To conclude, we offer some practical and conceptual considerations in a brief critical perspective on "glucolipotoxicity" as it relates to pancreatic β-cells pathophysiology in T2DM. These suggestions evolved over the course of our studies using a tissue culture model with isolated pancreatic mouse and human islets, which resulted in the present study and a related report that is yet to be published. The term "glucolipotoxicity" refers in the present T2DM connection to the fact that development of β-cell damage in tissue culture requires chronic exposure (continued or frequently repeated for 3–5 days) to unphysiologically high levels of both glucose (8–16 mM) and fatty acids (a nominally 0.25–0.5 mM 2:1 mixture of oleic and palmitic acid at about 1.5% albumin). To demonstrate the existence of a "glucolipotoxic" state in isolated islets, it is absolutely required that glucose (optimally above threshold levels at 8 mM) be present in the functional test system (i.e. dynamic fuel stimulated insulin secretion including its neuro-entero-endocrine potentiation and glucose dependent stimulation of insulin release by FA or a physiological amino acid mixture). Our lab has developed and partially characterized (although not yet published) "lipotoxicity resistant" glucose-free islet culture and test

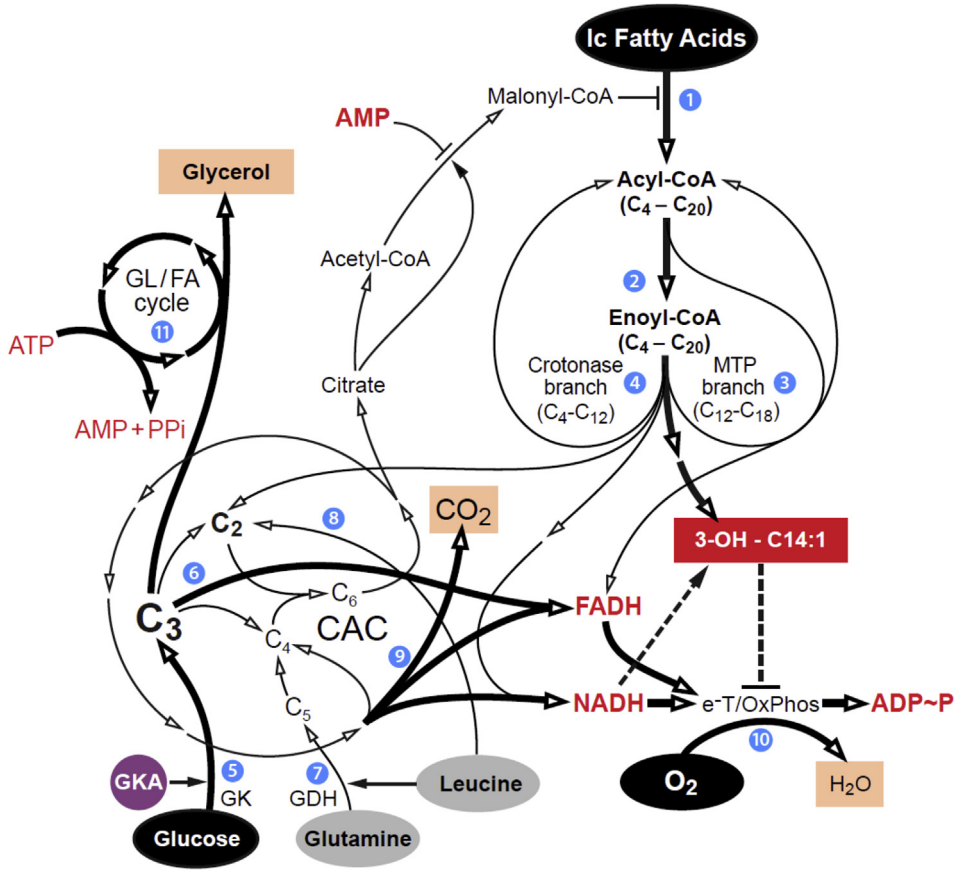

Figure 7: Generation of 3-hydroxy-tetradecenoic acid as mediator of pancreatic β-cell bioenergetics failure. Long-chain acyl-CoA is transferred into mitochondria via the carnitine cycle with CPT-1/2 and the AC transpoter (**1**) and is oxidized to 2-enoyl-CoAs by three chain length specific acyl-CoA DHs (**2**). There are two parallel, chain length specific, pathways for metabolism of enoyl-CoAs: a) via the mitochondrial trifunctional protein (*MTP branch* (**3**)), catalyzing the entire sequence of reactions for long-chain acids; and b) via a sequence of three medium/short-chain enzymes (crotonase, hydroxyacyl-CoA DH and ketoacyl-CoA thiolase) (the *crotonase branch* (**4**)). At high concentrations of palmitoyl CoA/oleoyl-CoA, the pathway is overloaded [29]: its flux drops and metabolites accumulate due to competition between acyl CoAs of different chain lengths for a set of acyl-CoA DHs with overlapping substrate specificity, resulting in *accumulation of CoA-esters, in case of islet tissue of 3-hydroxytetradecenoic acid (3-OH-C14:1), which inhibits/uncouples oxidative phosphorylation* (**10**). Glucose synergizes in 3-OH-C14:1 accumulation by increasing the mitochondrial [NADH]/[NAD^+] ratio and inhibiting 3-hydroxyacyl-CoA DH in the MTP (**3**) and crotonase (**4**) branches. Acetyl-CoA (C2) and reductive cofactors (**6** and **9**) are generated by FA oxidation and also from glucose (**5**) glutamine (Q) or leucine (L) (**7**). High glucose (**5**) or amino acids (**7**) increase anaplerosis of the citric acid cycle (CAC, **8**) and efflux of citrate, oxaloacetate, malate, aspartate from mitochondria associated with an energy wasting proton leak into the mitochondria via UCP-2. Cytosolic citrate is converted into the CPT-1 regulator malonyl-CoA, tightly controlled by 5′AMP and citrate. Glucose is a far more powerful reductant of NAD^+ and FAD (the latter via the α-glycero-P shuttle) than Q plus L, explaining, in part, its critical contribution to lipotoxicity as a result of the unbridled feeder enzyme and glucose sensor glucokinase (GK, **5**). High Glucose also enhances the glycerolipid/FAs cycle (**11**), an energy wasting process. Note that the possible participation of peroxysomal lipid metabolism is not considered here for lack of reliable information and for the sake of simplicity.

systems, which should be highly useful for evaluating hypothetical mechanisms. Thus, defined operationally, "glucolipotoxicity" is indeed a clearly reproducible, experimental phenomenon. However, whether this is a realistic duplication of the in vivo condition leading to the development of T2DM, which would warrant wide use in studies to elucidate underlying molecular mechanisms, can and continues to be debated. For example, it needs to be acknowledged that beta-cell dysfunction and loss, the hallmark of T2DM, usually does not occur by an increased glucose and fatty acid load, which realistically exists, in genetically uncompromised obese or physically inactive individuals with profound insulin resistance. The endocrine pancreas of such individuals is not damaged; rather, it adapts to the fuel overload. Perhaps it needs to be acknowledged that the commonly used tissue culture model of glucolipotoxicity is not ideal. Commonly used culture models use isolated islets from normal, genetically uncompromised mouse strains or humans and exposes the islets to extreme, often insufficiently defined, conditions for 3—5 days. Obviously, this is not an ideal system to study primary molecular mechanisms that result in β-cell dysfunction and loss, which are characteristic of T2DM. Still, numerous and diverse molecular mechanisms have been described and proposed as the cause of β-cell damage in T2DM using this approach, including, as we suggest here, that the accumulation of an hydroxylated unsaturated fatty acid is an expression of glucolipotoxicity and the cause of β-cell dysfunction. At best, one might defend that mechanisms uncovered this way could be involved in worsening dysfunction and hastening the loss of mass of pancreatic β-cells in individuals with genetic risk for T2DM, rendering them incapable of adapting to increased functional demand for more insulin. Experimental studies using the present tissue culture model of glucolipotoxicity could be improved if isolated islets from diabetes prone mouse strains or isolated islets of normoglycemic first degree relatives of families with high incidence of T2DM were used with less severe glucolipotoxic culture conditions in order to better duplicate the human realities of this disease.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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