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Reversibility of Metabolic and Morphological Changes Associated With Chronic Exposure of Pancreatic Islet β-Cells to Fatty Acids

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ABSTRACT

Pancreatic β-cells metabolise both lipid and glucose nutrients but chronic exposure (>24 h) to elevated fatty acid (FA) concentrations results in deleterious metabolic and morphological changes. The aims of this study were to assess the adaptive morphological, metabolic and secretory responses of islet β-cells to exposure and removal of FA. Isolated mouse islets and INS-1 β-cells were exposed to oleate or palmitate (0.5 mM) or a 1:1 mixture of both FA for 48 h prior to a 24 h period without FA. Subsequent changes in lipid storage and composition (triglycerides, TG and phospholipids, PL), gene expression, β-cell morphology and glucose-stimulated insulin secretion (GSIS) were determined. Intracellular TG content increased during exposure to FA and was lower in cells subsequently incubated in FA-free media ($P < 0.05$); TG storage was visible as oil red O positive droplets (oleate) by light microscopy or 'splits' (palmitate) by electron microscopy. Significant desaturation of β-cell FA occurred after exposure to oleate and palmitate. After incubation in FA-free media, there was differential handling of specific FA in TG, resulting in a profile that tended to revert to that of control cells. FA treatment resulted in elevated lipolysis of intracellular TG, increased FA oxidation and reduced GSIS. After incubation in FA-free media, oxidation remained elevated but inhibition of FA oxidation with etomoxir (10 μM) had no effect on the improvement in GSIS. The β-cell demonstrates metabolic flexibility as an adaptive response to ambient concentrations of FA. J. Cell. Biochem. 109: 683–692, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ISLET; REVERSIBILITY; LIPOLYSIS; TRIGLYCERIDE; PHOSPHOLIPIDS; OXIDATION; ADIPOSE DIFFERENTIATION-RELATED PROTEIN; PALMITOLEATE

The importance of glucose metabolism in pancreatic islet β-cell metabolism and secretion is well recognised but although plasma fatty acids (FA) are essential for the normal functioning of the β-cell [Stein et al., 1996; Dobbins et al., 1998] their role in β-cell metabolism in health and disease is less well understood. Exposure to elevated FA results in changes in the expression of many β-cell genes associated with stimulated insulin secretion [Kelpe et al., 2003; Olofsson et al., 2007] and lipid metabolism [Bikopoulos et al., 2008] which could reflect an adaptation to the change in nutrient metabolism. The anaplerotic generation of malonyl-CoA in the presence of FA is believed to inhibit the mitochondrial FA transporter carnitine palmitoyl transferase-1 (CPT-1), partitioning FA away from oxidative pathways and towards esterification [Roduit et al., 2004]. In combination with elevated glucose concentrations, prolonged exposure to FA causes the expansion of intracellular TG stores in human islets [Berne, 1975a; Lupi et al., 2002] and clonal β-cells [Segall et al., 1999; Moffitt et al., 2005]. Subsequent hydrolysis of these lipid stores by lipase action [Mulder et al., 1999; Fex and Mulder, 2008] is likely to increase substrate availability for FA oxidation and may thus compete with glucose oxidation leading to impaired insulin secretion [Zhou and Grill, 1994; Rubi et al., 2002]. Cellular TG droplets are usually associated with proteins of the PAT family (perilipin, adipophilin and tail-interacting protein of 47 kDa) which act in concert with lipases to regulate the lipolysis of intracellular TG. In the β-cell, adipophilin or adipose differentiation-related protein (*Adfp*) gene expression has

Abbreviations used: ADFP, adipose differentiation-related protein; CPT-1, carnitine palmitoyl transferase-1; FA, fatty acids; DGAT, diacylglycerol *O*-acyltransferase 1; GCK, glucokinase; GSIS, glucose-stimulated insulin secretion; ORO, oil-red O; PL, phospholipid; PPAR, peroxisome proliferator-activated receptor; SCD-1, stearoyl CoA desaturase-1; TG, triglyceride; TSIS, tolbutamide-stimulated insulin secretion; UCP-2, uncoupling protein 2.

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been reported [Kharroubi et al., 2006] but the localisation of this protein has not been demonstrated.

We have recently demonstrated increased infiltration of adipocytes within the exocrine pancreas and increased storage and altered pancreatic triglyceride (TG) composition in mice maintained on a high-fat diet, [Pinnick et al., 2008] and this can also be seen in obese humans [Olsen, 1978; Tushuizen et al., 2007]. This suggests that in obese individuals, even though the concentration of fasting plasma non-esterified FA may not always be elevated [Reeds et al., 2006], locally released FA could expose the β-cell to considerably higher FA concentrations than would be predicted from systemic concentrations. Prolonged exposure of pancreatic islets to elevated concentrations of FA reduces insulin secretion in vitro [Lupi et al., 2004; Moffitt et al., 2005; Olofsson et al., 2007] and has been implicated in the declining insulin secretory capacity of the β-cell which accompanies the onset of type 2 diabetes [Kashyap et al., 2003; Pankow et al., 2004]. There is limited evidence to suggest that the removal of exogenous FA results in the recovery of stimulated insulin secretion in vitro [Zhou and Grill, 1994; Boucher et al., 2004]. Pharmacological reduction of plasma non-esterified FA has been shown to improve β-cell function in individuals at risk of type 2 diabetes [Paolisso et al., 1998].

Glucose-regulated TG/FA cycling in the β-cell has been proposed as a potential mechanism for the generation of lipid-derived signalling molecules required for stimulus-secretion coupling of insulin (long chain acyl-CoA esters (Lc-CoA), diacylglycerol (DAG) and ceramide) [Nolan et al., 2006]. The composition of β-cell TG may therefore be of particular importance for glucose-stimulated insulin secretion (GSIS). Following prolonged exposure to FA, β-cell lipid stores not only expand but are compositionally remodelled to incorporate predominant FA from the surrounding 'milieu' [Dobbins et al., 2002; Moffitt et al., 2005]. The incorporation of exogenous FA into β-cell phospholipids (PL) [Ramanadham et al., 2000; Moffitt et al., 2005] may also impact on β-cell function by influencing membrane fluidity, receptor functionality or fusion pore expansion [Olofsson et al., 2007].

Our aim was to examine changes in β-cell nutrient metabolism associated with FA exposure and removal in mouse islets and INS-1 cells. Cells chronically exposed (48–72 h) to palmitate, oleate or palmitate/oleate mixture (1:1) were cultured for an additional 24 h in the absence of exogenous FA. Lipid metabolism and composition, cell morphology, insulin secretion and mRNA expression of some relevant genes were evaluated.

MATERIALS AND METHODS

ISLET ISOLATION AND CELL CULTURE CONDITIONS

Mouse islets were isolated from NMRI mice, aged 6–20 weeks, using collagenase digestion and cultured overnight (5% CO_2, 37°C) free-floating in RPMI-1640 medium (Sigma-Aldrich, UK) containing 11.1 mmol/L glucose and supplemented with 10% foetal calf serum, 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Principles of good laboratory animal care were followed and procedures were carried out according to institutional and UK Home Office guidelines. Cells were cloned out from an original batch of INS-1E to give a robust cell line which was more glucose-sensitive for insulin secretion. These cells are referred to as INS-1 cells since they no longer had the original phenotype. Cells were maintained in RPMI-1640 as previously described [Moffitt et al., 2005]. Stock solutions of oleate and palmitate (10 mmol/L) were prepared as previously described [Moffitt et al., 2005] and diluted to a final concentration of 0.5 mmol/L (complexed to 0.5% BSA) in glucose-free RPMI-1640 (Gibco, UK) supplemented with 16 mmol/L glucose (islets) or 20 mmol/L glucose (INS-1 cells). For insulin secretion, electron microscopy and real-time PCR studies, 6×10^5 INS-1 cells were seeded into 6-well plates (Sarstedt, UK); for morphological observations 1×10^5 cells were seeded into 8-well Permanox plastic slides (Gibco). Cells or islets were cultured for 48–72 h in control medium (containing 0.5% BSA) or in FA medium, they were washed in Dulbecco's phosphate-buffered saline (Sigma-Aldrich) and cultured for a further 24 h in control medium (0.5% BSA and (islets) 16 mmol/L or (INS-1) 20 mmol/L glucose) with no added FA. For FA oxidation studies, etomoxir sodium hydrate (Sigma-Aldrich) was added to the media (10 μmol/L) 1 h prior to transfer to FA-free media. There was no evidence of a substantial loss of INS-1 or islet cells due to lipid-induced apoptosis [Kharroubi et al., 2004]. Protein measurements of cultured INS-1 cells were similar under control and FA cultured conditions (control 38.0 ± 1.09; oleate 38.5 ± 1.39; palmitate 36.5 ± 1.04 μg protein/well). Apoptotic nuclei in islets and INS-1 cells were quantified by EM; 100 cells were examined per sample and the number of apoptotic nuclei compared. A similar morphological analysis was made of the occurrence of 'splits' in cells cultured with or without palmitate.

LIPID STORAGE AND MOBILISATION

TG and PL content and composition of INS-1 cells were analysed by gas chromatography [Moffitt et al., 2005] after (1) 72 h exposure to FA or (2) after 48 h FA treatment and a 24 h FA-free period. The differences in TG concentration between (1) and (2) were calculated for each fatty acid species and expressed as a relative difference in TG-FA content. To assess lipolysis, media was collected at the end of the final 24 h period of culture (with or without FA) and glycerol (Randox Laboratories Ltd, UK) and FA concentrations (WAKO NEFA C kit, Alpha Laboratories, UK) were analysed enzymatically using culture media as a blank. TG content, glycerol and FA concentrations were expressed relative to cellular protein (Bradford assay). To assess FA oxidation, $^{14}CO_2$ production was measured using a modified protocol [Pease and Wiggins, 1999]. Cells were incubated at 37°C in glucose-free RPMI-1640 supplemented with 3 mmol/L glucose, 0.5 mmol/L oleate and 0.02 MBq [1-^{14}C] oleate (1,908 MBq/mmol; Amersham). After 1 h, glass 'boats' containing 1 ml of 3 mol/L KOH were inserted inside the flasks in order to capture CO_2. After 2 h at 37°C, 1 ml of 60% perchloric acid was injected into the flask in order to liberate CO_2 in the medium. Flasks were resealed and placed on a shaker at room temperature for 2 h. The KOH was collected and trapped $^{14}CO_2$ was measured by liquid scintillation counting on a Beckman LS6500 scintillation counter.

CELL MORPHOLOGY

Lipid accumulation and cellular morphology were assessed by light microscopy in INS-1 cells stained with Oil Red O (ORO) and by electron microscopy as previously described [Moffitt et al., 2005]. Proteins of the PAT family were identified in INS-1 cells by immunolabelling with antibodies for ADFP and perilipin [Pinnick et al., 2008] and visualised with goat anti-rabbit FITC (Vector, USA) and fluorescence microscopy.

TABLE I. TG-FA Content and Composition (pmol/μg protein)

	Control (72 h)	Oleate (72 h)	Oleate (48 h +FA-free)	Palmitate (72 h)	Palmitate (48 h +FA-free)
16:0	67.5 ± 5.2	138.6 ± 13.5	119.6 ± 19.1	$222.8 \pm 40.3^{*}$	$43.7 \pm 6.3^{\ddagger}$
16:1n-7	5.0 ± 5.0	$69.7 \pm 1.4^{*}$	$9.3 \pm 5.7^{\dagger}$	17.6 ± 5.1	5.9 ± 5.9
18:0	26.4 ± 0.5	34.7 ± 15.2	27.3 ± 6.2	31.0 ± 7.4	21.6 ± 3.8
18:1n-9	37.1 ± 10.4	$904.9 \pm 43.3^{*}$	$110.4 \pm 14.6^{\dagger}$	20.4 ± 4.8	37.6 ± 9.0
Other	30.6 ± 30.1	175.5 ± 53.7	89.6 ± 33.5	36.2 ± 23.2	45.0 ± 25.3
Total	166.5 ± 29.4	$1323.3 \pm 85.7^{*}$	$356.3 \pm 47.7^{\dagger}$	328.0 ± 48.9	153.8 ± 30.6

'Other' was composed of FA accounting for less than 5% of total FA and included; 20:1n-9, 20:2n-6, 22:0, 22:1n-9, 22:6n-3 ($P < 0.05$; *condition vs. control; †condition vs. oleate; ‡condition vs. palmitate).

REAL-TIME PCR

INS-1 cells were harvested in Tri reagent (Sigma-Aldrich) and RNA was extracted using Ambion's RNAqueous-Micro system (Ambion Ltd, Huntingdon, UK). RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Germany), and RNA purity was assessed using the 260/280 nm ratio on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). cDNA was synthesised from 2 μg of RNA with the SuperScript III First-Strand synthesis system (Invitrogen, Paisley, UK). Reactions were run in quadruplicate with predesigned primer/probe sets (Assays-on-Demand, Applied Biosystems, USA) for insulin I (*Ins1*) (Rn 02121433_g1), insulin II (*Ins2*) (Rn01774648_g1), *Cpt1* (Rn 00580702_m1), *Ucp2* (Rn00571166_m1), *Pparα* (Rn00566193_m1), *Gck* (Rn00561265_m1), *Adfp* (Rn01472318_m1), *Scd1* (Rn00594894_g1) and *Dgat* (Rn00584870_m1). Analysis was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). Expression values were calculated by the efficiency-corrected C_t model ($Q = \text{Efficiency}^{[\text{minimum}C_t - \text{sample}C_t]}$) [Pfaffl, 2001]. All samples were normalised to the housekeeping genes, β-actin (Rn00667869_m1) and cyclophilin A (Rn00690933_m1) and are reported as the $\Delta\Delta C_t$ value.

INSULIN CONTENT AND SECRETION

After an initial incubation in Krebs–Ringer HEPES (KRH) containing 3 mmol/L glucose for 1 h, cells or batches of five islets were transferred to fresh KRH containing 3 mmol/L glucose for 45 min. This was followed by a 45 min stimulation period in KRH containing 20 mmol/L glucose (INS-1 cells) or 16 mmol/L glucose (islets) or 3 mmol/L glucose and 0.5 mmol/L tolbutamide. The media was collected at the end of each 45 min incubation for insulin measurements by ELISA (Diagenics, UK). The minimum detection limit was 0.15 μg/L and the intra-assay coefficient of variation was 5%. Insulin secretion data is presented as a stimulation index calculated as the ratio of insulin secreted under stimulated conditions to insulin secreted under basal conditions. Following secretion studies islets were collected in acid-alcohol (1% HCl, 70% alcohol) and sonicated for measurement of insulin content.

STATISTICAL ANALYSIS

Results are expressed as mean values of three or more experiments (±SEM). Data were analysed using SPSS for Windows v14 (SPSS UK, Chertsey, UK). Statistical significance was determined using Student's *t*-test and ANOVA with Bonferroni post hoc testing for multiple comparisons ($P < 0.05$).

RESULTS

REMODELLING OF INTRACELLULAR TG AND PL IN INS-1 CELLS

Total intracellular TG content was higher in INS-1 cells following FA treatment; TG in oleate-treated cells was sevenfold higher but a more modest increase in palmitate-treated cells was found (Table I). Culture for 48 h with FA followed by a 24 h FA-free period resulted in lower TG content with concentrations close to that of control cells cultured for the same time period in the absence of FA (Table I). The increase in TG was mainly due to incorporation of the specific experimental FA (Table I); the proportion of oleate increased 3-fold and palmitate 1.5-fold in the relevant test cells (Fig. 1A and Table SI, Supplementary Data) to make up 69% for oleate and 68% for palmitate respectively. Cells treated with either fatty acid accumulated palmitoleate ($P < 0.05$ for oleate-treated cells) whilst the amount of stearate remained relatively constant. The amount of oleate in the palmitate-treated cells did not increase, whereas the amount of palmitate in the oleate-treated cells increased significantly. After removal of the specific exogenous fatty acids, the accumulated species in TG did not disappear equally (Fig. 2), for example fractionally more palmitate was removed in the palmitate-treated cells (80% vs. 13%), resulting in significant remodelling of intracellular TG composition. Thus, in cells previously treated with palmitate the FA profile was actually similar to that of the control, although the proportion of oleate remained higher than controls in cells previously treated with oleate (Fig. 1A).

Total PL content (representing PL in the plasma membrane and all intracellular compartments) was unchanged by FA treatment but PL were enriched with the experimental FA and this effect was more pronounced in cells treated with oleate than palmitate (Fig. 1B and Table SII, Supplementary Data). Remarkably, the proportion of palmitoleate accounted for ~25% of total PL FA in palmitate-treated cells but following the FA-free period this accounted for only 12% which was not different from controls. In general, the PL FA profiles of cells subsequently cultured in the absence of FA tended to be similar to controls, although, in the case of cells previously treated with oleate there remained differences in the proportions of oleate and palmitoleate, and the proportion of oleate remained lower in palmitate cells.

INTRACELLULAR LIPID ACCUMULATION IN INS-1 CELLS AND MOUSE ISOLATED ISLETS

Light microscopy demonstrated lipid accumulation in INS-1 cells treated for 48 and 72 h with oleate which was visible as variable sized droplets (ORO-positive) in the cytoplasm (Fig. 3). These

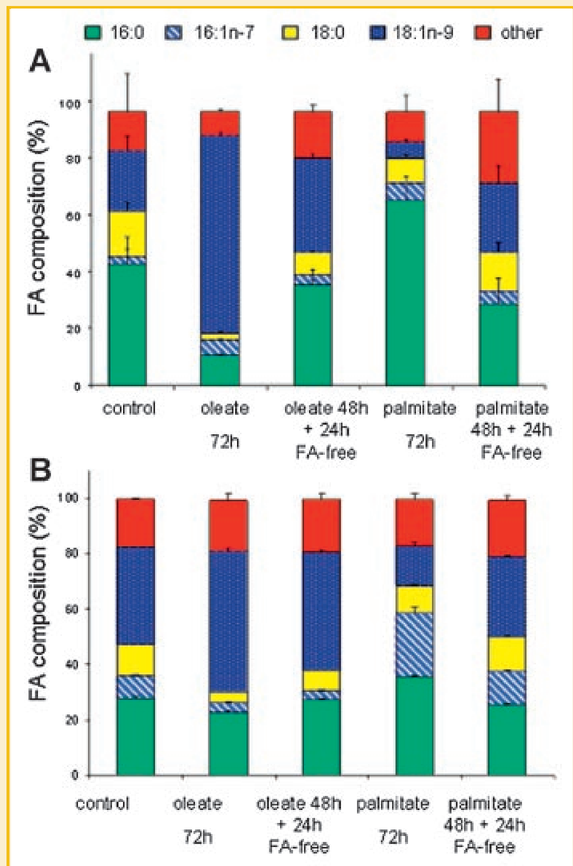

Fig. 1. FA composition of INS-1 TG and PL. Following 72 h FA exposure, incorporation of the specific experimental FA was observed in INS-1 TG (A), as determined by gas chromatography. Cells which were exposed to FA for 48 h followed by a 24 h FA-free period contained proportionally lower amounts of the experimental FA and the TG-FA profile was similar to that of control cells. PL were also enriched with the experimental FA following 72 h exposure (B). There was a greater incorporation of oleate into INS-1 PL than palmitate. The proportion of palmitoleate was greater in palmitate-treated cells than control cells. PL FA profiles of cells cultured without FA for 24 h were similar to controls but the proportion of palmitoleate remained higher in cells which had previously been exposed to palmitate.

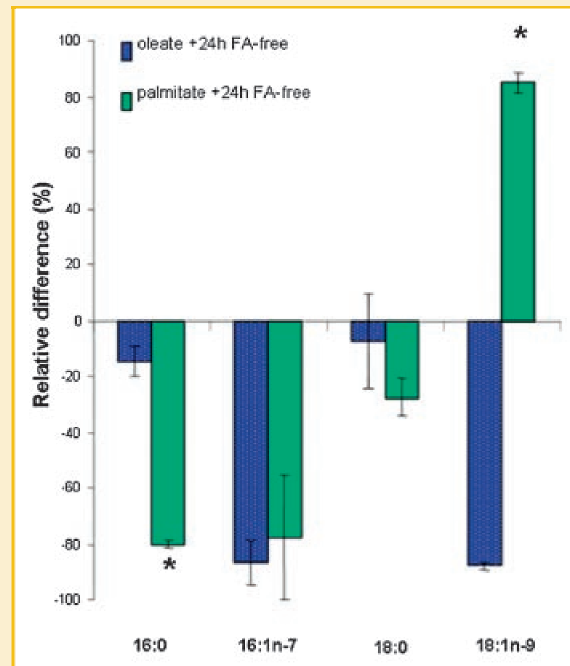

Fig. 2. Change in accumulated cellular TG following removal of FA from media. The TG composition of INS-1 cells was analysed after 72 h exposure to oleate and palmitate or after 48 h FA exposure and 24 h in FA-free media. The difference in TG concentration between the two culture conditions was calculated for each FA species and expressed as a relative difference in TG-FA content. Mean ± SEM, $P < 0.05$; *palmitate versus oleate treatment.

droplets were not present in the untreated cells (control) and very few droplets were present following the removal of FA from the culture. A diffuse ORO positivity was present in cells exposed to palmitate as described previously [Moffitt et al., 2005] but no droplets were found. Immunolabelling for the PAT family proteins identified ADFP but not perilipin in close association with intracellular lipid droplets in oleate-treated cells (Fig. 3) but is was not possible to see any association of ADFP in cytoplasmic lipid in cells exposed to palmitate.

Electron microscopy confirmed the presence of large lipid droplets in the cytoplasm of cells treated with oleate (Fig. 4c,d) which were not present in control cells (Fig. 4a,b). These were reduced in size and extent following the FA-free period (Fig. 4g). In palmitate-treated cells, instead of lipid droplets, large angular vacuoles or 'splits' were observed in the cytoplasm (Fig. 4e,f). These electron-lucent structures were in close proximity to the rough endoplasmic reticulum, which was severely distended and distorted. Cells in which the FA had been removed for the last 24 h showed little sign of 'splits'. Quantitation demonstrated that 89% of INS-1 cells had 'splits' following culture for 72 h with palmitate. In cells cultured with palmitate followed by a FA-free period, only 26% had visible 'splits'. The frequency of islet β-cells containing visible 'splits' was lower than that seen in INS-1 cells (48% of β-cells). Splits were also visible in some of the less frequently occurring islet cell types (14/15 glucagon-containing α-cells and 2/3 pancreatic polypeptide (PP) cells contained 'splits') following culture with palmitate. After the FA-free period, islets had less evidence of 'splits'; splits were only found in 35% of β-cells and not at all in α- or PP-cells.

LIPOLYSIS WAS DEPENDENT ON FA EXPOSURE

To explore the mobilisation of TG by lipolysis in INS-1 cells, FA and glycerol concentrations accumulating in the culture medium during the last 24 h of the culture period were measured. Glycerol concentrations were elevated twofold in treated cell media compared to media of cells not exposed to FA (controls) (Fig. 5A). It was not possible to measure endogenously derived FA in the presence of experimental FA in the media at 72 h culture. In cells cultured for 48 h with FA followed by a 24 h FA-free period, extracellular glycerol concentrations were significantly lower compared to cells continuously exposed to FA but were higher than controls. Under these conditions, FA concentrations in the media were also higher than controls (Fig. 5B). The addition of

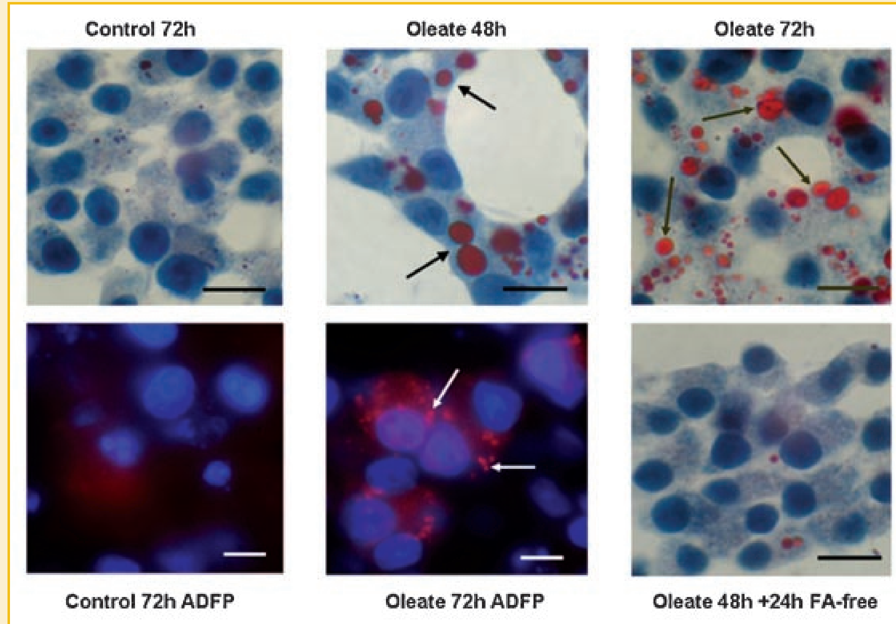

Fig. 3. Visualisation of changes of intracellular lipid stores in INS-1 cells by light microscopy. Lipid accumulation in INS-1 cells was examined with light microscopy. Cells cultured for 48–72 h with oleate contained numerous lipid droplets of different sizes which labelled red (arrows) with oil red O (ORO). Sections counterstained with haematoxylin. These intracellular droplets were rarely seen in control conditions. In cells exposed to oleate (48 h) followed by a 24 h period of FA-free culture, the number of ORO positive droplets was low. Immunolabelling for ADFP demonstrated positive labelling of droplets (arrows) of similar size and distribution to that seen with ORO in cells treated with oleate (72 h) but not under control conditions. Scale bars 15 μm.

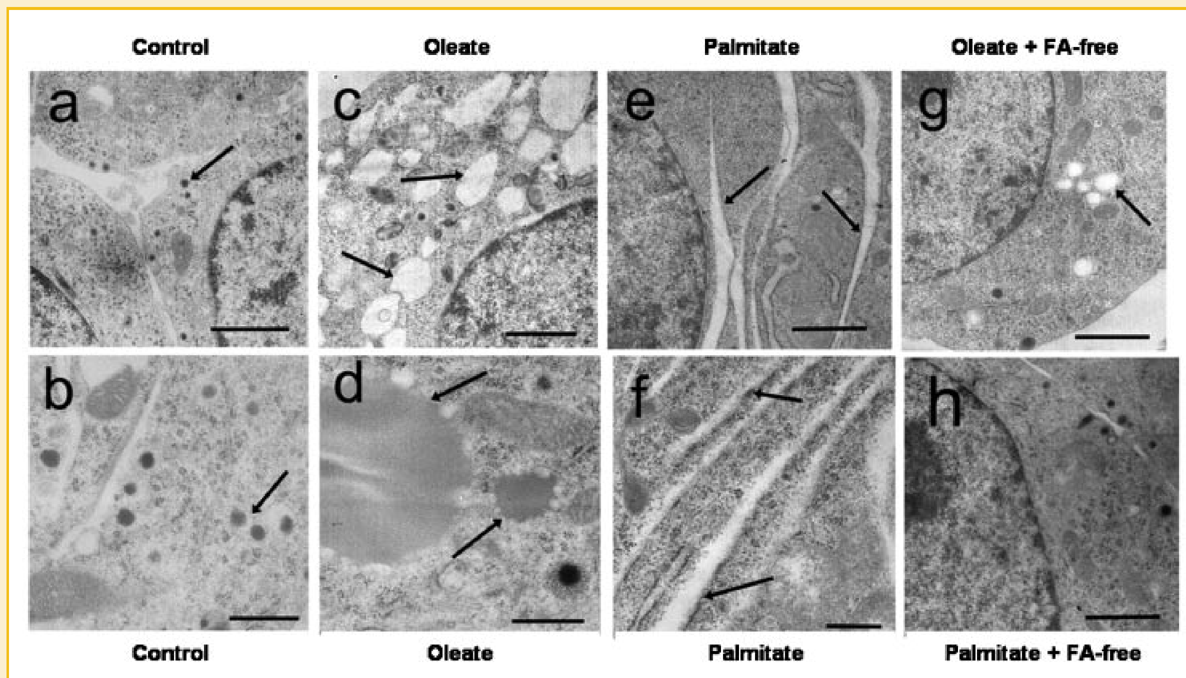

Fig. 4. Visualisation of changes of intracellular lipid stores in INS-1 cells by electron microscopy. Cells cultured for 72 h under control conditions (a,b) had numerous insulin secretory granules (arrows). Culture with oleate for 72 h (c) resulted in lipid droplets appearing as vacuoles (arrows) or (d) electron opaque material not bounded by a membrane in the cytoplasm (arrows). Cells cultured with palmitate for 72 h (e) had angular 'splits' associated with (f) dilated endoplasmic reticulum (ER) identified by a lining with ribosomes (arrows). Following oleate-free culture (g), the number and size of lipid droplets (arrows) was low and when palmitate was removed from the culture media (h), the ER 'splits' were largely absent. Scale bars (a,c,e,g,h) = 1 μm; (b,d,f) = 500 nm.

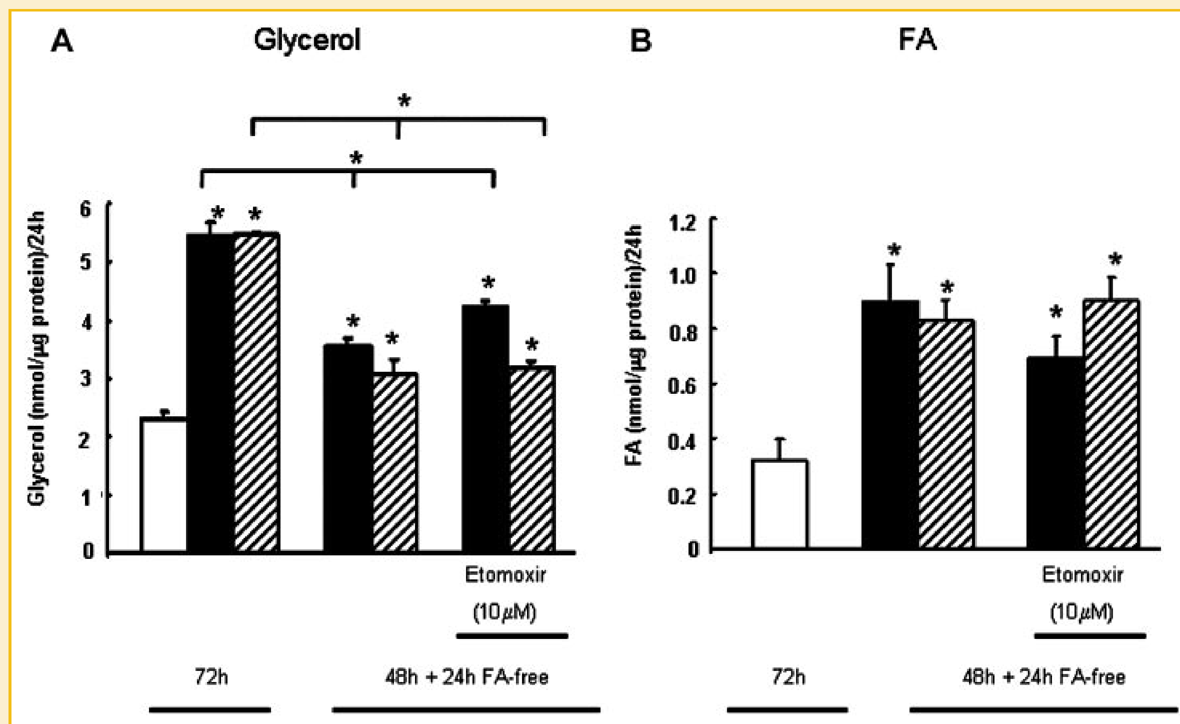

Fig. 5. Extracellular glycerol and FA concentrations. Glycerol in the media (A) was elevated in oleate (solid bars) and palmitate-treated cells (striped bars) compared to controls (open bars) following 72 h exposure. Cells cultured for 48 h with FA plus a 24 h FA-free period had lower concentrations of glycerol in the media compared to cells cultured continuously with FA but remained elevated compared to controls. The addition of etomoxir (10 μM) during the FA-free period had no effect on extracellular glycerol concentrations. B: Extracellular FA concentrations were elevated in cells cultured for 48 h with FA plus a 24 h FA-free period. Endogenously derived FA could not be measured in the media in the presence of exogenous FA. The addition of etomoxir during the FA-free period had no effect on extracellular FA concentrations ($P < 0.05$; *condition vs. control).

etomoxir during the FA-free period did not significantly increase either glycerol or FA release from cells previously treated with either oleate or palmitate.

FA OXIDATION WAS INCREASED BY FA EXPOSURE

Control cells produced 6.2 ± 1.1 pmol of $^{14}CO_2$/h and this was threefold higher in cells which were cultured continuously for 72 h with FA (Fig. 6). Following removal of the FA, cells treated for 48 h with oleate displayed significantly lower $^{14}CO_2$ production than cells exposed continuously to FA, but there was no change in $^{14}CO_2$ production in palmitate-treated cells. Etomoxir treatment in the last 24 h of the culture period had no effect on $^{14}CO_2$ production under control conditions but significantly reduced $^{14}CO_2$ production in cells previously exposed to FA (Fig. 6).

CHRONIC FA EXPOSURE ALTERS THE EXPRESSION OF SELECTED GENES ASSOCIATED WITH METABOLISM

Expression of *Cpt1* was lower in cells exposed to FA for 48 h followed by the FA-free period than cells which were continuously exposed to FA (Table II), but *Cpt1* expression in oleate-treated cells remained higher than controls. No significant changes were observed in the expression of other genes associated with lipid and glucose metabolism (*Pparα*, *Adfp*, *Dgat*, *Ucp2*, and *Gck*). Cells exposed to oleate followed by the removal of FA also displayed lower expression of *Ins1* compared to cells continuously exposed to oleate and a similar trend was observed in palmitate-treated cells. The addition of etomoxir during the FA-free period did not alter the expression of any of the genes investigated.

FA-IMPAIRED GSIS IN ISLETS AND INS-1 CELLS WAS REVERSIBLE

Exposure of islets to FA for 72 h resulted in impaired GSIS (Fig. 7; reduced by 59% with oleate, 70% with palmitate and 45% with palmitate/oleate (1:1); $P < 0.05$) and tolbutamide-stimulated insulin secretion (TSIS) (Fig. 7A). This was similar to the effects observed at 48 h (GSIS was reduced by 57% with oleate and 80% with palmitate; $P < 0.05$, data not shown). Islets which were exposed to FA for 48 h followed by the 24 h FA-free period, however, did not display significantly impaired GSIS or TSIS compared to control islets (Fig. 7A). In the case of GSIS, insulin secretion was significantly higher in islets cultured for 24 h in the absence of FA compared to those continuously exposed to FA. Insulin content of 72 h FA-treated islets was significantly reduced compared to controls (control 14.8 ± 2.41; oleate 9.6 ± 2.05; palmitate 5.87 ± 0.94; oleate/palmitate (1:1) 9.23 ± 0.31 ng/islet; $P < 0.05$), but was equivalent to the insulin content of control islets after the 24 h FA-free period (oleate 14.9 ± 2.89; palmitate 12.3 ± 2.11; oleate/palmitate 12.4 ± 0.81 ng/islet). No change in basal insulin secretion at 3 mmol/L glucose was observed in FA-treated islets (control 13.5 ± 2.77; oleate 15.5 ± 1.54; palmitate 11.7 ± 1.11 ng/islet).

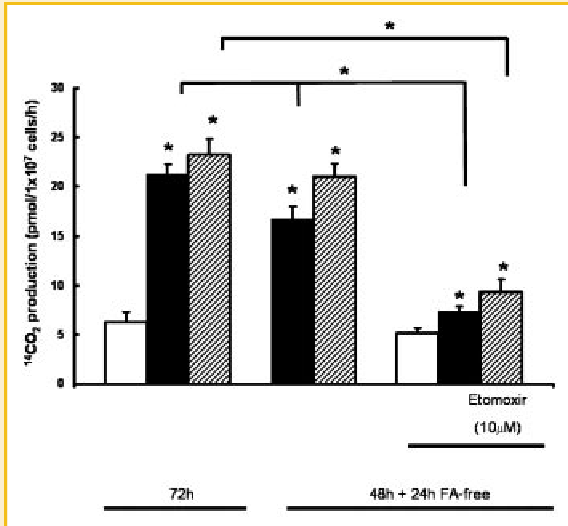

Fig. 6. β-cell FA oxidation. FA oxidation was determined in oleate and palmitate-treated cells or following the 24 h FA-free period, by the measurement of $^{14}CO_2$ release. Cells exposed to FA for 72 h (oleate, solid bars; palmitate, striped bars) displayed a three- to fourfold increase in $^{14}CO_2$ release compared to control cells (open bars). Cells cultured with FA for 48 h plus a 24 h FA-free period also had elevated concentrations of released $^{14}CO_2$. When etomoxir (10 μM) was added prior to the 24 h FA-free period $^{14}CO_2$ release was reduced to levels comparable to that of controls demonstrating the efficacy of this concentration of the CPT-1 inhibitor ($^*P < 0.05$). The addition of etomoxir did not affect $^{14}CO_2$ release under control conditions.

INS-1 cells (Fig. 7C) displayed a similar trend to islets (Fig. 7A) following 72 h exposure to FA, however, the magnitude of GSIS and the effects of FA were markedly lower than seen in islets. Specifically, the culture of islets with oleate caused a 2.5-fold reduction in GSIS while only a 1.3-fold reduction was seen in INS-1 cells. In INS-1 cells, impaired GSIS was more marked with palmitate treatment (58%) compared to oleate (35%) and higher GSIS was observed in cells recovering from FA treatment (Fig. 7C). To determine whether elevated FA oxidation during the FA-free period affected the improvement of insulin secretion, GSIS was measured following 24 h exposure to etomoxir. Etomoxir treatment did not significantly affect GSIS (oleate, 1.70 ± 0.10 vs. oleate + etomoxir, 1.40 ± 0.11; palmitate, 1.12 ± 0.01 vs. palmitate + etomoxir 1.04 ± 0.30 stimulation index ± SEM).

DISCUSSION

We have demonstrated that β-cell FA metabolism is able to respond and adapt to chronic exposure to FA in our islet and INS-1 cell models. On exposure to palmitate, there was significant FA desaturation, particularly in the PL pool. On exposure to oleate, there was significant accumulation of palmitoleate in cellular TG, suggesting de novo synthesis of palmitate followed by desaturation. We have previously reported that in mice the fatty acid composition of the whole pancreas is consistent with the occurrence of de novo lipogenesis [Pinnick et al., 2008]. When extracellular FA were removed for 24 h there was an almost complete reduction of accumulated TG and rapid remodelling of TG and PL FA composition towards that observed in control conditions. Lipolysis of intracellular TG and oxidation of FA, which were higher than seen in control conditions, remained high following the removal of FA, accounting for the lower TG content. These metabolic changes were accompanied by improvements in insulin secretion and content which were not dependent on FA oxidation.

In regulating TG stores, changes in the composition of β-cell FA occurred beyond a simple accumulation and subsequent loss of the test fatty acids. Firstly, there is strong evidence that desaturation of fatty acids accompanied the accumulation of TG. Palmitoleate is a product of the desaturation of palmitate via SCD1 [Nakamura and Nara, 2004] and there was significant accumulation of palmitoleate in TG of oleate-treated cells. The proportion of palmitoleate was also very high in the PL of the palmitate-treated cells (23% vs. 8% in control cells). These findings were surprising, given that we found expression of *Scd1* to be undetectable. Expression of *Scd1* in INS-1 cells has previously been reported to be undetectable in some [Lai et al., 2008] but not all studies [Ramanadham et al., 2002]. Mice lacking SCD1 have demonstrated that SCD1 is active in a distinct

TABLE II. Changes in Gene Expression in INS-1 Cells Following the Removal of Exogenous FA

	Control (72 h)	Oleate (72 h)	Oleate (48 h +FA-free)	Oleate (48 h +FA-free (etomoxir))	Palmitate (72 h)	Palmitate (48 h +FA-free)	Palmitate (48 h +FA-free (etomoxir))
Lipid metabolism							
Cpt1	0.34 ± 0.13	0.96 ± 0.08*	0.65 ± 0.09*†	0.75 ± 0.05*	0.92 ± 0.04*	0.60 ± 0.09‡	0.62 ± 0.11‡
Pparα	1.05 ± 0.07	0.86 ± 0.20	0.78 ± 0.05	0.72 ± 0.07	1.02 ± 0.21	0.70 ± 0.08	0.76 ± 0.08
Adfp	1.00 ± 0.18	1.01 ± 0.19	1.02 ± 0.17	0.96 ± 0.25	0.89 ± 0.13	0.99 ± 0.16	1.10 ± 0.16
Dgat	1.10 ± 0.06	0.93 ± 0.18	0.90 ± 0.11	0.94 ± 0.14	1.00 ± 0.21	0.90 ± 0.12	1.05 ± 0.16
Scd1	ND	ND	ND	ND	ND	ND	ND
Glucose metabolism							
Ucp2	0.70 ± 0.04	0.87 ± 0.01	0.75 ± 0.05	0.85 ± 0.13	0.82 ± 0.03	0.70 ± 0.05	0.79 ± 0.06
Gck	0.66 ± 0.05	0.83 ± 0.08	0.69 ± 0.06	0.74 ± 0.08	0.79 ± 0.07	0.64 ± 0.05	0.76 ± 0.06
Insulin secretion							
Ins1	0.64 ± 0.12	1.04 ± 0.11*	0.70 ± 0.09†	0.76 ± 0.09	1.03 ± 0.04*	0.75 ± 0.06	0.76 ± 0.05
Ins2	0.45 ± 0.07	1.17 ± 0.17*	0.72 ± 0.11†	0.81 ± 0.09†	0.74 ± 0.05*	0.64 ± 0.12	0.65 ± 0.09

ND, not detected.
ΔΔCt values normalised to β-actin and cyclophilin A ($P < 0.05$; *condition vs. control; †condition vs. oleate; ‡condition vs. palmitate).

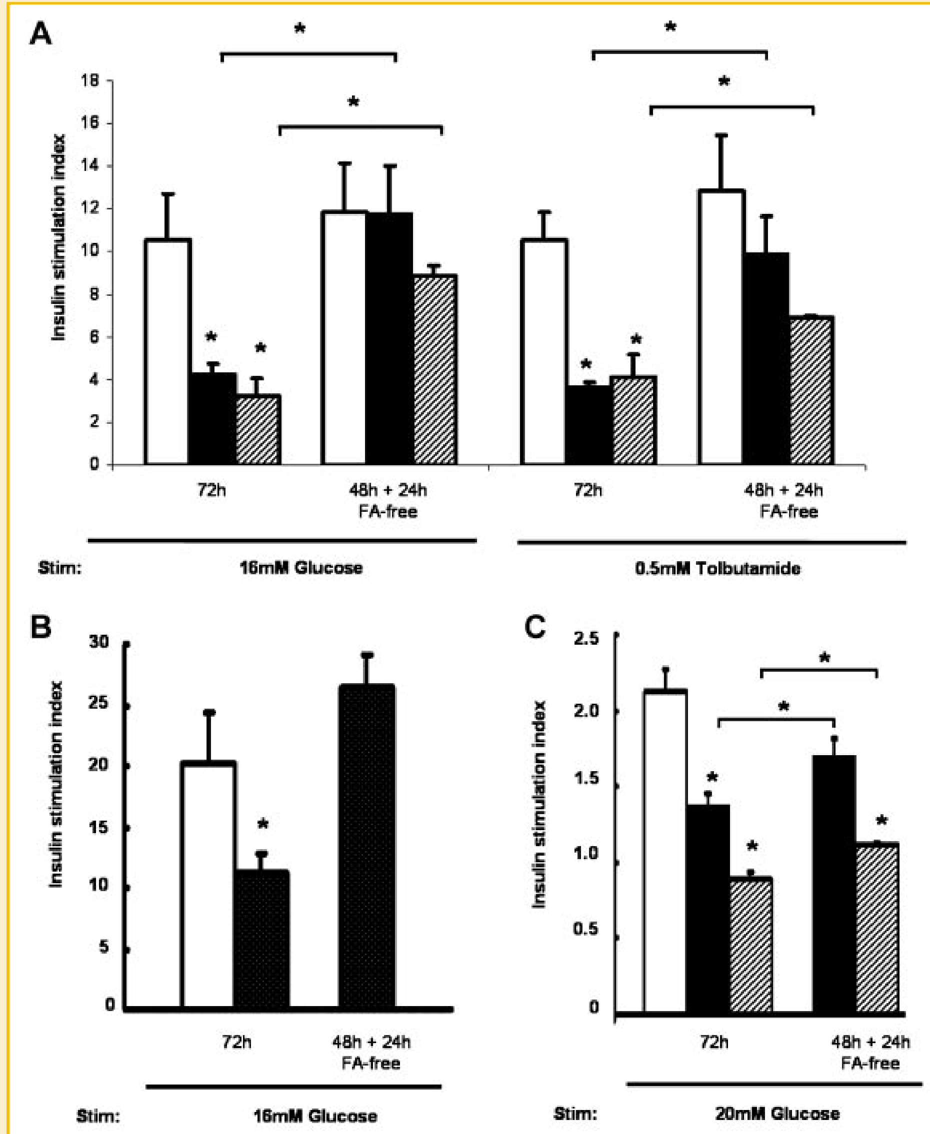

Fig. 7. Recovery of FA-impaired stimulated insulin secretion in islets and INS-1 cells. Islets exposed to FA for 72 h (A) displayed significantly impaired GSIS (16 mM glucose) and TSIS (0.5 mM tolbutamide). Following 48 h exposure to oleate (solid bars) or palmitate (striped bars) and an additional 24 h culture in the absence of FA, GSIS and TSIS were no longer different from control islets (open bars) indicating the recovery of stimulated insulin secretion. Islets exposed to a 1:1 mixture of oleate/palmitate (spotted bar) for 72 h (B) displayed impaired GSIS. There was no significant difference in GSIS between islets cultured for 48 h with FA plus a 24 h FA-free and 72 h control islets (open bar). INS-1 cells exposed to oleate (solid bar) or palmitate (striped bar) for 72 h (C) displayed impaired GSIS (20 mM glucose). Cells cultured in oleate (solid bars) for 48 h plus a 24 h FA-free period displayed no significant difference in GSIS compared to 72 h control cells (open bar) and GSIS was higher than in cells continuously exposed to oleate. GSIS was impaired in cells treated with palmitate (striped bars) for 48 h plus a 24 h FA-free period compared to control cells but was significantly higher than in cells continuously exposed to palmitate ($^*P < 0.05$; $n = 3$).

pancreatic islet population in vivo: in those studies, islet TG contained less palmitoleate [Flowers et al., 2007]. In the mouse, four SCD isoforms have been identified which display a tissue-specific expression profile [Flowers and Ntambi, 2009]. Our findings do not rule out the possibility of the presence of an alternative SCD isoform in the rat-derived INS-1 cells. We did not find statistically significant desaturation of palmitate-TG in the palmitate-treated cells. This may have been because of selective incorporation of palmitoleate into PL. Accordingly, we did not find desaturation of stearate (18:0) in TG but after removal of exogenous FA, the proportion of oleate was higher in PL than in the presence of FA, despite no difference in concentration of total PL. This suggests an adaptive response to maintain the proportion of unsaturated fatty acids in the PL pool, probably an important factor for maintaining membrane fluidity and cell viability. Indeed, no change in membrane fluidity has recently been reported in mouse islets exposed to palmitate for 72 h (Hoppa et al., 2009). Whilst it is evident that high concentrations of saturated FA can be cytotoxic in vitro [Welters et al., 2004; Moffitt et al., 2005] we did not observe this in the current study. In accordance with our findings, Busch et al. have demonstrated that β-cells, shown to be resistant to palmitate-induced cytotoxicity, display 2.8-fold higher *Scd1*

expression [Busch et al., 2005]. The desaturation of palmitate to palmitoleate in our INS-1 cells could therefore account for the lack of palmitate-induced cytotoxicity in our experiments.

After removal of exogenous fatty acids, there was a remarkable disappearance of both test fatty acids that had accumulated in TG. Eighty percent of accumulated palmitate and 88% of accumulated oleate were metabolised within 24 h. For other fatty acids, there was variable removal, suggesting either selective lipolysis or esterification of fatty acids from the cellular FA pool. Thus the composition of TG and PL fatty acids in cells previously exposed to fatty acids tended to return to that of control cells. In the case of cells which had been exposed to palmitate the reversal of TG accumulation was particularly striking, given the dramatic and extensive nature of the morphological changes observed. The palmitate-rich TG was situated in cytoplasmic 'splits', previously described to be largely composed of solid tripalmitin [Moffitt et al., 2005] whereas oleate-rich TG (after exposure to oleate) was stored in variably sized cytoplasmic lipid droplets. After the removal of FA, cytoplasmic splits and lipid droplets were largely absent, in agreement with the biochemical measurements. Although hormone sensitive lipase (*Hsl*) is expressed in β-cells [Mulder et al., 1999], findings from *Hsl* knockout mice [Fex et al., 2004] have suggested the presence of other β-cell lipases, such as adipose triglyceride lipase (ATGL) [Fex et al., 2006], which may also contribute to lipolysis in the β-cell. We found lipid droplets to be closely associated with the regulatory lipid droplet protein, ADFP, which may interact with β-cell lipases in a similar manner to the HSL–perilipin interaction characterised in adipocytes [Clifford et al., 2000]. Both oleate and palmitate-rich TG stores were lipolysed when exogenous FA were removed from the media, as demonstrated by the release of FA and glycerol. However, glycerol release was actually greater in the presence of the test FA than when FA were absent which is consistent with the concept of FA recycling in the β-cell [Nolan and Prentki, 2008].

The β-cell has been reported to have a high capacity for FA oxidation; of the total amount of palmitate taken up by islets, 50% is oxidised and 50% esterified [Berne, 1975b]. However, elevated FA oxidation has been associated with impaired insulin secretion in INS-1 cells [Brun et al., 1997; Rubi et al., 2002]. We observed a modest improvement in insulin secretion which was apparent within 24 h of removing FA from INS-1 cells. This was consistent with previous findings [Zhou et al., 1996] and a mixture of oleate/palmitate (1:1) produced similar results. The fact that FA oxidation remained elevated in the absence of FA in our study could explain why only a small improvement in insulin secretion was observed. However, when FA oxidation was inhibited by etomoxir treatment, the improvement in insulin secretion was still observed to a similar extent which would indicate that the recovered GSIS occurred independently of a change in FA oxidative status. This is in contrast to the findings of Zhou and Grill [1994] who reported partial reversal of FA-impaired GSIS in rat islets when FA oxidation was inhibited with etomoxir.

In conclusion, it is evident that the β-cell has a large but labile capacity for intracellular TG storage and exhibits the potential to metabolically and morphologically adapt to changes in FA nutrient supply. Visceral obesity which is associated with ectopic fat deposition in many tissues such as the liver, muscle and pancreas [Kovanlikaya et al., 2005; Larson-Meyer et al., 2006] is likely to alter the FA milieu of the pancreatic β-cell leading to exposure to high concentrations of FA and impaired β-cell function. Weight loss through lifestyle management has been shown to be beneficial in reducing liver fat [Leclercq and Horsmans, 2008]. It is not yet clear whether pancreatic fat would respond in the same way, but it could be hypothesised that a reduction in pancreatic fat as a result of weight loss may ultimately lead to reduced exposure of β-cells to FA with improvements in β-cell function.

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