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## Mitochondrial uncoupling protein-2 is not involved in palmitate-induced impairment of glucose-stimulated insulin secretion in INS-1E insulinoma cells and is not needed for the amplification of insulin release



Verena Hirschberg Jensen, Charles Affourtit\*

School of Biomedical and Healthcare Sciences, Plymouth University, Drake Circus, Plymouth PL4 8AA, UK

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### ABSTRACT

We have recently shown that overnight exposure of INS-1E insulinoma cells to palmitate in the presence of high glucose causes defects in both mitochondrial energy metabolism and glucose-stimulated insulin secretion (GSIS). Here we report experiments designed to test the involvement of mitochondrial uncoupling protein-2 (UCP2) in these glucolipotoxic effects. Measuring real-time oxygen consumption in siRNA-transfected INS-1E cells, we show that deleterious effects of palmitate on the glucose sensitivity of mitochondrial respiration and on the coupling efficiency of oxidative phosphorylation are independent of UCP2. Consistently, palmitate impairs GSIS to the same extent in cells with and without UCP2. Furthermore, we knocked down UCP2 in spheroid INS-1E cell clusters (pseudoislets) to test whether or not UCP2 regulates insulin secretion during prolonged glucose exposure. We demonstrate that there are no differences in temporal GSIS kinetics between perfused pseudoislets with and without UCP2. We conclude that UCP2 is not involved in palmitate-induced impairment of GSIS in INS-1E insulinoma cells and is not needed for the amplification of insulin release. These conclusions inform ongoing debate on the disputed biochemical and physiological functions of the beta cell UCP2.

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### 1. Introduction

Pancreatic beta cells secrete insulin in response to an increase in the blood glucose level [1]. Mitochondria play an important role in this glucose-stimulated insulin secretion (GSIS) as they generate signals during oxidative glucose catabolism that trigger and amplify insulin release [2]. In the canonical model of GSIS, glucose breakdown causes an increase in the cytoplasmic ATP/ADP ratio, which leads, sequentially, to the inhibition of  $K_{ATP}$  channels, depolarisation of the plasma membrane potential, opening of voltage-gated calcium channels, calcium influx, and the exocytosis of insulin-containing granules [1]. Whilst this triggering sequence of events accounts for GSIS in the short term, less well understood amplifying mechanisms are necessary to sustain insulin release for a prolonged period of time [3]. The glucose sensitivity of the ATP/ADP ratio is a characteristic of pancreatic beta cells that reflects the extraordinary bioenergetics of these cells: whilst in most cell types

*Abbreviations:* BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; GSIS, glucose-stimulated insulin secretion; UCP, uncoupling protein; NEFA, non-esterified fatty acid

\* Corresponding author. Tel.: +44 1752 584649; fax: +44 1752 584605.

E-mail address: [charles.affourtit@plymouth.ac.uk](mailto:charles.affourtit@plymouth.ac.uk) (C. Affourtit).

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the ATP/ADP poise is controlled exclusively by ATP turnover, in beta cells this ratio is largely governed by glucose oxidation, i.e. ATP supply [4]. This unusual control structure of energy metabolism allows regulation of the ATP/ADP ratio – and hence insulin secretion – by the coupling efficiency of oxidative phosphorylation.

Pancreatic beta cells contain a mitochondrial uncoupling protein (UCP2, [5]) that, in analogy with the archetypal uncoupling protein UCP1 [6], is believed to dissipate the mitochondrial protonmotive force as heat and thus uncouple oxidative phosphorylation [7]. Recent work on proteoliposomes has revealed, however, that reconstituted UCP2 protein exhibits specific substrate exchange activity, which suggests UCP2 exports carbon skeletons from the mitochondrial matrix [8]. Given the unresolved molecular function of UCP2, it is perhaps not surprising that the protein's physiological role in beta cells has not yet been established conclusively either. Depending on their genetic background, global *Ucp2* knockout mice exhibit either improved glucose tolerance and GSIS [5,9] or unaltered glucose tolerance [10] and impaired GSIS [9]. Beta-cell-specific ablation of UCP2 leads to glucose-intolerant mice whose pancreatic islets, however, exhibit higher GSIS than their wild type counterparts [11]. These discrepant observations have led to different functional models that predict a pathological role for UCP2 in beta cell failure and

consequent development of Type 2 diabetes on the one hand [12], and a physiological role in protecting cells against oxidative stress on the other [13]. Biochemical studies with INS-1E insulinoma cells support the possibility that, by dampening the generation of glucose-induced mitochondrial reactive oxygen species, UCP2 acutely attenuates GSIS and, in the long term, prevents oxidative stress [14,15]. Informed by effects on mitochondrial coupling efficiency and GSIS in INS-1E cells, we previously proposed a role for UCP2 in regulating the beta cells' physiological response to fluctuations in nutrient supply [16]. More specifically, we hypothesised that by partially uncoupling oxidative phosphorylation, UCP2 allows turnover of the tricarboxylic acid cycle beyond the control of the ATP/ADP ratio. Such uncontrolled turnover would ensure production of mitochondrial GSIS amplification signals that are necessary to sustain insulin secretion when nutrient levels, and hence the ATP/ADP ratio, are high [16].

Recently, we have shown that palmitate impairs GSIS in INS-1E cells when administered overnight at high glucose [17]. This glucolipotoxic phenotype coincides with mitochondrial defects: palmitate decreases the glucose sensitivity of mitochondrial respiration and also lowers coupling efficiency of oxidative phosphorylation [17]. Palmitoleate, i.e., palmitate's monounsaturated counterpart, does not exert deleterious effects on GSIS and mitochondrial energy transduction, but does not protect against palmitate-provoked damage either [17]. Palmitate-induced defects are indeed reminiscent of how UCP2 affects the mitochondrial bioenergetics and GSIS of INS-1E cells [14,15]. In line with mouse and islet studies reported by others [18,19], it is thus conceivable that UCP2 mediates the mitochondrial respiratory dysfunction and associated GSIS impairment caused by palmitate in INS-1E cells [17]. This hypothesis has not yet been tested in insulinoma cells.

In this paper we report studies that were designed to test (i) whether or not UCP2 mediates the detrimental effects of palmitate on oxidative phosphorylation and GSIS in INS-1E cells and (ii) if UCP2 is needed to sustain insulin secretion during prolonged glucose exposure. Via an RNAi approach we show that palmitate disturbs mitochondrial respiration and GSIS in a similar way, qualitatively and quantitatively, in INS-1E cells with and without UCP2. Effects of palmitate on the bioenergetics of INS-1E cells  $\pm$  UCP2 are consistent with the lack of UCP2 influence on the GSIS phenotype. Furthermore, we reveal that spheroid INS-1E cell clusters (pseudoislets [20,21]) exhibit temporal GSIS kinetics that are also independent of UCP2. We conclude that UCP2 is not responsible for palmitate-induced GSIS impairment in INS-1E insulinoma cells and is not required for the amplification of insulin release.

## 2. Materials and methods

### 2.1. Tissue culture

INS-1E cells were donated by Prof. Noel Morgan (University of Exeter Medical School) and maintained according to [22] in RPMI-1640 growth medium that contained 11 mM glucose and was supplemented with 5% (v/v) foetal bovine serum, 10 mM Hepes (pH 7.4), 1 mM sodium pyruvate, 50 U/mL penicillin, 50 mg/mL streptomycin, 500 mM  $\beta$ -mercaptoethanol and 2 mM glutamax (Catalogue #35050-061, Life Technologies). To facilitate the formation of pseudoislets,  $3 \times 10^6$  INS-1E cells were added in 7.5 mL RPMI to 75 cm<sup>2</sup> suspension culture flasks (Greiner bio-one #658195) that were coated with 1% (w/v) gelatin [21].

### 2.2. UCP2 knockdown and detection

UCP2 protein was knocked down in INS-1E cells via RNAi as described before [23] applying 200 nM scrambled or *Ucp2*-

targeted siRNA oligonucleotides (both from Ambion, Huntington, UK) and 1.67  $\mu$ g/mL Lipofectamine™ (Invitrogen, Paisley, UK). To generate UCP2-depleted pseudoislets, INS-1E cells were transfected immediately after adding them to gelatin-coated flasks using the same transfection agent and siRNA specifications as used for cells. After 24 h, pseudoislets were harvested by 2-min centrifugation at 200g, resuspended in fresh growth medium and then cultured for another 4 days before insulin secretion was measured. UCP2 protein depletion in pseudoislets was confirmed by Western blotting analysis as described before [24]. To prepare samples for this analysis, pseudoislets were spun down by 1-min centrifugation at 1000g and then resuspended in 40  $\mu$ L ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1% (v/v) Nonidet P40, 0.25% (w/v) sodium deoxycholate, 0.1% (v/v) SDS, 150 mM NaCl, 1 mM EDTA and 500x diluted protease inhibitor (#P8340, Sigma-Aldrich). After vigorous shaking for 20 min at 4 °C, non-solubilised material was pelleted by 15-min centrifugation at 14,000g and 4 °C. Protein levels of the supernatants were quantified by bicinchoninic acid assay (Thermo Scientific 23227) and 50  $\mu$ g of protein was precipitated with ice-cold acetone. After 1-h incubation at  $-20$  °C, protein was pelleted and resuspended in loading buffer appropriate for polyacrylamide gel electrophoresis and subsequent Western blotting analysis [24].

### 2.3. Fatty acid exposure

The non-esterified fatty acids (NEFAs) palmitate and palmitoleate were added in conjugation to fatty-acid-free bovine serum albumin (BSA, Sigma-Aldrich A7030) as described in [17]. Transfected and non-transfected INS-1E cells were exposed to NEFAs as described in [24]. Control cells were exposed to BSA alone.

### 2.4. Bioenergetics

Mitochondrial oxygen consumption was measured in INS-1E cells using a Seahorse XF24 extracellular flux analyser as described before [17]. Glucose sensitivity of mitochondrial respiration was calculated as glucose-stimulated oxygen consumption normalised to basal respiratory activity, whilst the coupling efficiency of oxidative phosphorylation was defined as the oligomycin-sensitive part of glucose-stimulated mitochondrial respiration [23]. Absolute respiratory activities were determined by normalising oxygen uptake rates to INS-1E cell number. Cell densities were determined by fluorescent DAPI-staining as described in [24].

### 2.5. Static GSIS

Insulin secretion was measured in INS-1E cell monolayers as described in [17]. Measuring static insulin secretion in INS-1E pseudoislets, the monolayer protocol was adapted to deal with the relatively high mobility of spheroid cell clusters. Pseudoislets were 'hand-picked' under a stereotactic microscope using a P20 pipettor and were transferred to a V-bottomed 96-well plate (Greiner bio-one #651101) dispensing 15 cell clusters per well in a total volume of 10  $\mu$ L growth medium. Pseudoislets were starved from glucose by adding 90  $\mu$ L glucose-free RPMI (Sigma # R1383) supplemented with 1% (v/v) foetal bovine serum, 10 mM Hepes (pH 7.4), 50 U/mL penicillin, 50 mg/mL streptomycin, 500 mM  $\beta$ -mercaptoethanol and 2 mM glutamine. Pseudoislets were washed once and then incubated in this starvation medium for 2 h at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Subsequently, cell clusters were washed into glucose-free Krebs-Ringer-Hepes buffer (KRH) comprising 135 mM NaCl, 3.6 mM KCl, 10 mM Hepes (pH 7.4), 0.5 mM MgCl<sub>2</sub>, 0.75 mM CaCl<sub>2</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM glutamine and 0.1% (w/v) BSA, and then incubated in this buffer for 30 min at 37 °C under air. At this point, 80  $\mu$ L supernatant was removed for deter-

mination of basal insulin secretion. This volume was replaced immediately by KRH containing 28 mM glucose and clusters were incubated for another 30 min at 37 °C under air. At this point, 80  $\mu$ L supernatant was taken for determination of glucose-stimulated insulin secretion. All collected samples were spun for 5 min at 14,000 g (4 °C) to pellet carried-over cells, and supernatants were stored at –80 °C. Insulin was assayed by ELISA (Merckodia, Uppsala, Sweden) using mouse insulin as a standard. To measure DNA content, pseudoislets were resuspended in a total volume of 250  $\mu$ L lysis buffer (see *UCP2 detection*) and shaken vigorously for 5 min at 4 °C. Solubilised samples were stored at –80 °C until DNA level was determined with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life technologies #P7589) according to the manufacturer's instructions using the high range standard curve. Insulin secretion by INS-1E pseudoislets was normalised to DNA content.

## 2.6. Temporal insulin secretion kinetics

Pseudoislets were harvested from a single 75 cm<sup>2</sup> flask by 2-min centrifugation at 200 g and were resuspended in 220  $\mu$ L KRH with 200  $\mu$ M glucose. Based on [21], 190  $\mu$ L pseudoislet suspension was pipetted into a Swinnex 13 filter holder (Millipore) fitted with a 0.1  $\mu$ m nylon mesh (Plastok associates). KRH with 200  $\mu$ M glucose was pumped continuously through 3 pseudoislet-containing parallel chambers at a flow rate of 0.5 mL min<sup>-1</sup> using a peristaltic pump (Gilson Minipuls 3, peristaltic tubing orange yellow, ID 0.51 mm) and silicone tubing (Corning medical grade, ID: 1.98 mm, OD: 3.18 mm). The perfusion setup was placed in a bench top incubator (Stuart SI60D) to maintain a constant temperature of 37 °C. Following a 30-min wash, perfusate was collected in 2-min intervals for 50 min. Glucose and KCl levels of the perfusion buffer were altered as described in the legends of Figs. 4 and 6. Perfusate fractions were stored on ice and carried-over cells were spun down after the experiment by 1-min centrifugation at 1000g. Supernatants were either stored at –80 °C or assayed directly for insulin as above.

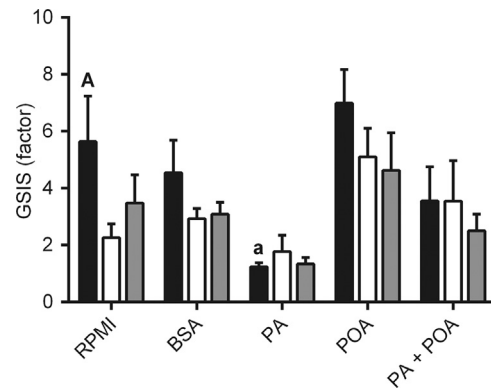
## 2.7. Statistics

Differences between means were tested for statistical significance by ANOVA as specified in the figure legends using GraphPad Prism Version 6.0 for Mac OS X (GraphPad software, San Diego, CA, USA).

## 3. Results

### 3.1. UCP2 does not mediate NEFA effects on GSIS

Transfection of INS-1E cells with *Ucp2*-targeted siRNA oligonucleotides results in UCP2 depletion to a level below the detection limit of our Western blotting assay [24]. Since cells transfected with scrambled siRNA contain the same UCP2 protein level as non-transfected cells [24], and since UCP2 knockdown is not affected by NEFA exposure [24], we deemed this RNAi approach suitable for exploring the involvement of UCP2 in palmitate-induced GSIS impairment. Non-transfected INS-1E cells grown in serum-containing medium and then starved from glucose 2 h before the GSIS assay, increase their insulin secretion rate almost 6-fold when subjected to 28 mM glucose (Fig. 1). GSIS in INS-1E cells transfected with scrambled siRNA is less pronounced, as insulin secretion is increased only just over 2-fold at 28 mM glucose in these cells. Not statistically significant but in line with our previous data [14,15], cells transfected with *Ucp2*-targeted siRNA exhibit higher GSIS than their scrambled-siRNA-transfected equivalents when grown in serum-containing medium. GSIS is

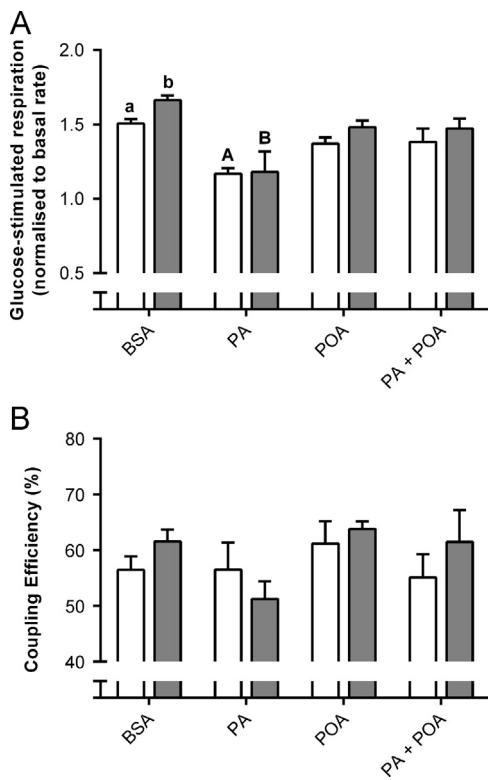


**Fig. 1.** Effect of NEFAs on GSIS in INS-1E cells  $\pm$  UCP2. GSIS was calculated as the fold increase in insulin secretion exhibited by glucose-starved cells in response to 28 mM glucose. GSIS was measured in non-transfected cells (black bars) and in cells transfected with scrambled or *Ucp2*-targeted siRNA (white and grey bars, respectively). Before the GSIS assay, cells were incubated in fully supplemented medium (RPMI), or were exposed for 24 h in serum-deprived medium to BSA-conjugated NEFAs (PA=palmitate, POA=palmitoleate, PA+POA=palmitate and palmitoleate) or to BSA alone. Bars represent means  $\pm$  SEM of 5 separate experiments in which each condition was measured 3 times. Statistical significance of mean differences was tested by 2-way ANOVA with Dunnett's multiple comparisons post-hoc analysis: A differs from a ( $P < 0.01$ ).

not affected significantly by serum deprivation (Fig. 1, BSA), neither in non-transfected nor in transfected cells, although the UCP2 phenotype appears to have been annulled by small non-significant effects. In agreement with our recent results [17], overnight palmitate exposure at 11 mM glucose almost completely abolishes GSIS in non-transfected cells (Fig. 1). Palmitate also dampens GSIS in transfected cells but the effects are relatively modest and not statistically significant ( $P=0.99$  and  $0.33$  for cells transfected with scrambled and *Ucp2*-targeted siRNA, respectively), likely because they emerge against a background of transfection-provoked stress (Fig. 1). Palmitate lowers GSIS to a similar level in transfected and non-transfected cells and, importantly, UCP2 knockdown does not alter this GSIS impairment significantly. Unlike palmitate, the monounsaturated palmitoleate does not cause any GSIS defects (Fig. 1). Instead, palmitoleate potentiates GSIS in transfected and non-transfected cells, although not to a statistically significant extent ( $P=0.71$ ,  $0.13$  and  $0.81$  for non-transfected, scrambled-transfected and *Ucp2*-targeted cells, respectively). This stimulatory effect is reminiscent of the significant GSIS increase observed in INS-1E cells exposed to palmitoleate at 4 mM glucose [17]. Importantly, the apparent GSIS potentiation does not require UCP2 (Fig. 1). The deleterious palmitate effect on GSIS is not significantly ameliorated by palmitoleate in cells  $\pm$  UCP2 (Fig. 1). UCP2 knockdown [14] and NEFA exposure [26] may lead to changes in insulin content that could account, in principle, for the observed GSIS phenotypes (Fig. 1). Irrespective of the exact mechanism, our data demonstrate that UCP2 does not influence the NEFA effects on GSIS.

### 3.2. UCP2 does not account for NEFA effects on oxidative phosphorylation

We have shown before that mitochondrial oxygen uptake in INS-1E cells cultured at 11 mM glucose, and then incubated in the absence of glucose 30 min before the respiratory assay, typically increases about 2-fold when cells are subjected to 11–28 mM glucose [17]. We have also shown that the mitochondrial respiratory sensitivity to glucose of transfected cells is considerably lower: 'scrambled' control cells starved from glucose for 2 h before the assay increase their oxygen uptake rate by a mere 1.3-fold when 30 mM glucose is added [15], whilst cells lacking UCP2



**Fig. 2.** NEFA effects on oxidative phosphorylation in INS-1E cells  $\pm$  UCP2. The sensitivity of mitochondrial respiration to 28 mM glucose (panel A) and coupling efficiency of glucose-stimulated oxidative phosphorylation (panel B) were determined in cells transfected with scrambled or *Ucp2*-targeted siRNA (white and grey bars, respectively). Before the respiratory assay, cells were incubated for 24 h in serum-deprived medium and exposed to BSA-conjugated NEFAs (PA=palmitate, POA=palmitoleate, PA+POA=palmitate and palmitoleate) or to BSA alone. Bars represent means  $\pm$  SEM of 4–5 separate experiments in which each condition was measured 3–4 times. Statistical significance of mean differences was tested by 2-way ANOVA with Sidak's multiple comparisons posthoc analysis: A differs from a ( $P < 0.01$ ) and B differs from b ( $P < 0.001$ ).

respond a little more strongly and show a 1.7-fold respiratory increase [15]. Consistently, in the experiments reported here, serum-deprived INS-1E cells transfected with scrambled siRNA increase their mitochondrial respiratory activity 1.5-fold in response to 28 mM glucose (Fig. 2A). This glucose-sensitivity is a little higher in UCP2-depleted cells, which increase mitochondrial respiratory activity approximately 1.7-fold (Fig. 2A). Palmitate-exposed cells only increase mitochondrial respiration about 1.2-fold when glucose is added (Fig. 2A), which is a significantly lower response than that of BSA control cells. The response by palmitate-exposed cells is of the same magnitude as we have seen before [17] and, importantly, does not depend on UCP2. Palmitoleate, administered alone or with palmitate, does not significantly change the glucose-sensitivity of mitochondrial respiration in cells with or without UCP2 (Fig. 2A).

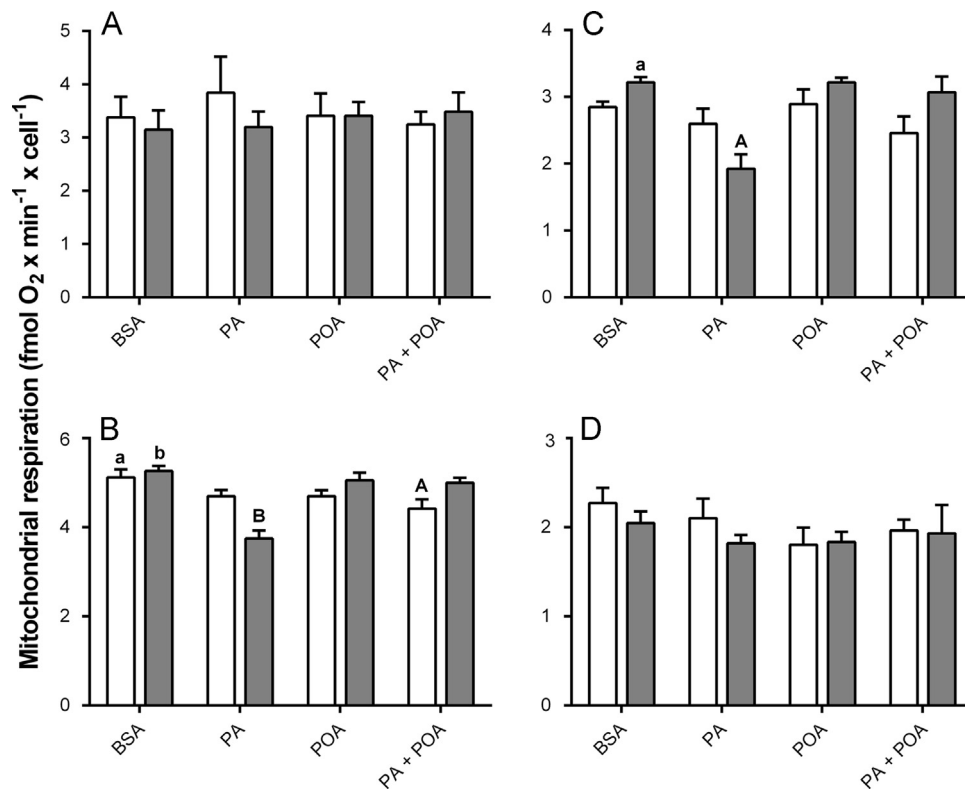
Compared to our previous experiments [17], NEFA effects on coupling efficiency of oxidative phosphorylation are relatively small, and they are indeed statistically not significant (Fig. 2B). Consistent with previous results [15], however, UCP2 knockdown tends to increase coupling efficiency in serum-deprived BSA control cells subjected to high glucose (Fig. 2B). Coupling efficiency of scrambled-transfected cells (Fig. 2B, BSA) is similar to the efficiency of non-transfected cells [17] but is relatively insensitive to palmitate (Fig. 2B, PA). In UCP2-depleted cells, palmitate tends to dampen coupling efficiency (Fig. 2B,  $P=0.16$ ). Palmitoleate, added alone or with palmitate, does not change coupling efficiency irrespective of UCP2 (Fig. 2B).

In line with our recent observations [17] overnight NEFA exposure at 11 mM glucose does not affect the absolute respiratory activity of INS-1E cells incubated without glucose (Fig. 3A). The lack of significant NEFA effect on this basal respiration is apparent in INS-1E cells that either contain or lack UCP2 (Fig. 3A). UCP2 depletion lowers the absolute basal respiratory rate in BSA control and palmitate-exposed cells, but these minor decreases are statistically insignificant (Fig. 3A). UCP2 knockdown does not affect absolute mitochondrial oxygen consumption stimulated by 28 mM glucose (Fig. 3B). Unlike non-transfected INS-1E cells [17], glucose-stimulated respiration is relatively insensitive to palmitate in cells transfected with scrambled siRNA (Fig. 3B) but, interestingly, UCP2 depletion augments palmitate sensitivity significantly (Fig. 3B). Palmitoleate lowers absolute glucose-stimulated respiration somewhat in scrambled-transfected cells, but this effect is only statistically significant when palmitoleate is added together with palmitate (Fig. 3B). In *Ucp2*-transfected cells, absolute glucose-stimulated respiration is insensitive to palmitoleate (Fig. 3B). Cells lacking UCP2 thus exhibit marginally higher glucose-stimulated mitochondrial respiratory rates after palmitoleate exposure ( $\pm$  palmitate) than cells with UCP2 (Fig. 3B).

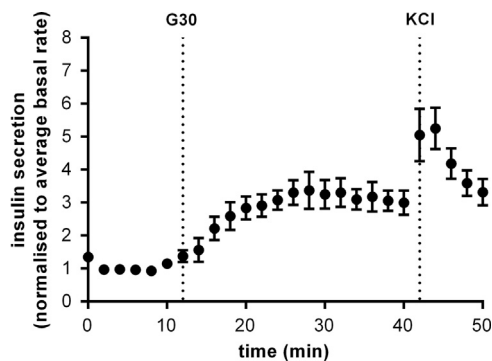
The trends we saw for glucose-stimulated respiration (Fig. 3B) are echoed by oligomycin-sensitive respiration, i.e., by the absolute mitochondrial oxygen consumption that is used to produce ATP (Fig. 3C). A marginal respiratory rate-lowering effect of palmitate in scrambled-transfected cells is significantly exacerbated by UCP2 knockdown, and oxygen uptake after palmitoleate exposure ( $\pm$  palmitate) tends to be a little higher in cells without than with UCP2 (Fig. 3C). Notably, BSA control cells exhibit comparably high absolute oligomycin-sensitive oxygen consumption after UCP2 knockdown (Fig. 3C). Palmitate also tends to decrease the oligomycin-resistant respiration, i.e., the absolute oxygen uptake that is associated with proton leak across the mitochondrial inner membrane in cells  $\pm$  UCP2 (Fig. 3D,  $P=0.87$  and  $0.78$ , respectively). Although not significant, these relatively small effects on proton leak are worth notice as they were not observed in non-transfected cells [17] and help to explain the relatively modest effect of palmitate on coupling efficiency of transfected cells (Fig. 2B): the dampening palmitate effect on respiration linked to ATP synthesis (Fig. 3C) that lowers coupling efficiency, is (partly) compensated by a concomitant lowering effect on proton leak (Fig. 3D). As the case for basal respiration (Fig. 3A), UCP2 knockdown lowers proton leak activity in BSA control and palmitate-exposed cells a little but not to a significant extent (Fig. 3D). Like palmitate, palmitoleate appears to lower oligomycin-insensitive respiration in cells with UCP2 ( $P=0.56$  and  $0.22 \pm$  palmitate, respectively) and without UCP2 ( $P=0.96$  and  $0.80 \pm$  palmitate, respectively) although variation between experiments is high (Fig. 3D).

### 3.3. UCP2 is not necessary for GSIS amplification

GSIS by pancreatic beta cells is a biphasic process: an initial short-term glucose-induced increase in insulin secretion is accounted for by the canonical GSIS triggering mechanism, whilst prolonged insulin release requires engagement of GSIS amplification pathways [3]. To test the notion that UCP2 may allow generation of mitochondria-derived GSIS amplification signals [2,16], we measured the temporal kinetics of insulin release by spheroid INS-1E cell clusters. The time-resolved data in Fig. 4 show that these INS-1E pseudoislets roughly triple their insulin secretion rate when subjected to glucose. Stimulated secretion is sustained for at least 30 min, which confirms that GSIS amplifying pathways are operational and agrees with INS-1E pseudoislet behaviour reported by others [21,22]. Similarly, the relatively sharp transient KCl-induced increase in insulin secretion is



**Fig. 3.** NEFA effects on absolute respiration in INS-1E cells  $\pm$  UCP2. Oxygen uptake was determined and normalised to cell number in INS-1E cells transfected with scrambled or *Ucp2*-targeted siRNA (white and grey bars, respectively). Before the respiratory assay, cells were incubated for 24 h in serum-deprived medium and exposed to BSA-conjugated NEFAs (PA=palmitate, POA=palmitoleate, PA+POA=palmitate and palmitoleate) or to BSA alone. During the respiratory assay, cells were incubated  $\pm$  28 mM glucose (panels B and A, respectively) and glucose-stimulated oxygen uptake was inhibited with 5  $\mu$ g/mL oligomycin to distinguish respiration coupled to ATP synthesis from respiration associated with proton leak (panels C and D, respectively). Bars represent means  $\pm$  SEM of 4–5 separate respiratory assays and cell densities to normalise respiration were determined from DAPI-fluorescence (cf. [24]) in 3–4 parallel experiments. Each condition was probed 3–4 times in all experiments. Statistical significance of mean differences was tested by 2-way ANOVA with Sidak's multiple comparisons posthoc analysis: A differs from a ( $P < 0.05$  and  $P < 0.0001$  in panels B and C, respectively) and B differs from b ( $P < 0.001$ ).



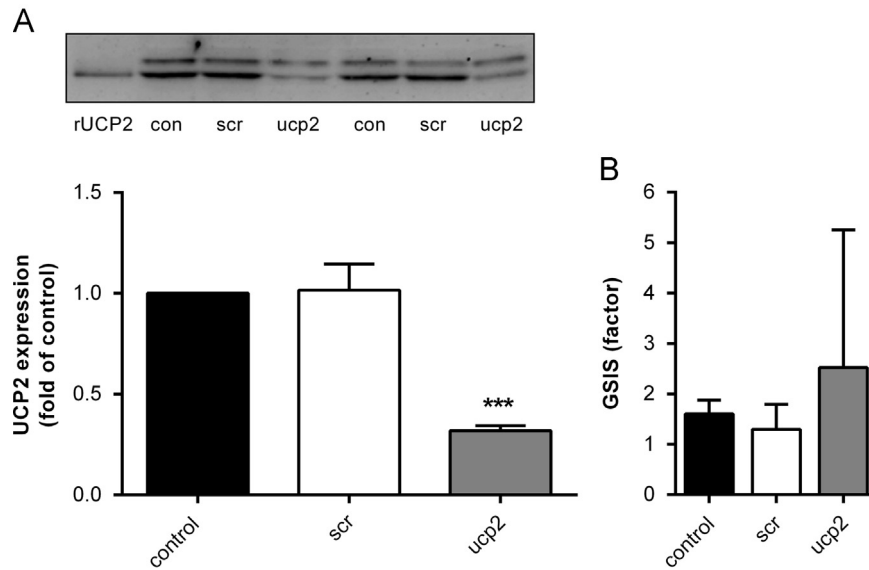
**Fig. 4.** Temporal kinetics of insulin secretion. Time-resolved insulin secretion by INS-1E pseudoislets was measured as described in “Section 2.6”. Pseudoislets were perfused with buffer containing 200  $\mu$ M glucose and then subjected, sequentially, to 30 mM glucose (G30) and 50 mM KCl at the times indicated by dotted lines. Insulin levels were determined in perfusate fractions taken at 2-min intervals, and insulin secretion was normalised to the average secretory rate observed before glucose addition. Data represent means  $\pm$  SEM of 7 independent experiments.

consistent with published results [22]. Like dispersed INS-1E cells [25], pseudoislets express a significant level of UCP2 protein (Fig. 5A). Transfection with *Ucp2*-targeted siRNA lowers the UCP2 level by almost 70%, whilst scrambled-transfected pseudoislets contain as much UCP2 protein as their non-transfected counterparts (Fig. 5A). Although variation is rather large, static insulin secretion experiments show that GSIS tends to be more pronounced in UCP2-depleted than in control pseudoislets (Fig. 5B,  $P=0.73$ ), an observation that is consistent with our INS-1E cell

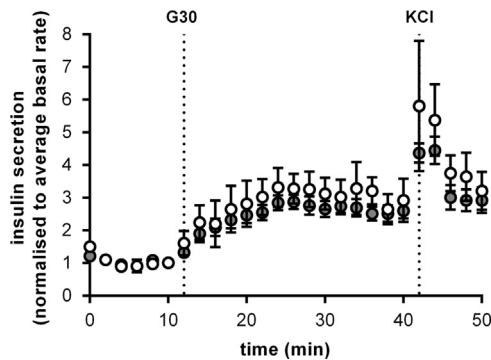
findings (Fig. 1A, [14,15]). Pseudoislets that were transfected with scrambled siRNA exhibit temporal GSIS kinetics (Fig. 6) that are similar to those observed in non-transfected cell clusters (Fig. 4), and the insulin secretory response to KCl is near-identical in these systems as well. Importantly, UCP2 depletion does not change the time-resolved insulin secretion profile to a statistically significant extent (Fig. 6), strongly suggesting that UCP2 is not needed to sustain GSIS. Similarly, deleterious palmitate effects on the insulin secretory profile of pseudoislets are not affected by UCP2 depletion (data not shown).

#### 4. Discussion

The involvement of UCP2 in pancreatic beta cell glucolipotoxicity remains controversial [26], largely because neither the biochemical nor the physiological function of this mitochondrial protein has been established conclusively at present. The suggested pathological model of UCP2 in development of Type 2 diabetes [12] predicts UCP2 mediates deleterious effects of fatty acids on beta cells, whilst the more recently proposed physiological role of UCP2 in protecting beta cells against oxidative stress [13] predicts UCP2 ameliorates such effects instead. The data reported here do not support either prediction. We demonstrate that UCP2 knockdown does not significantly affect palmitate-induced GSIS impairment in INS-1E cells (Fig. 1). This lack of UCP2 phenotype is echoed by the relative insensitivity of palmitate-induced mitochondrial respiratory defects to UCP2 depletion (Figs. 2 and 3). Moreover, we reveal that UCP2 activity



**Fig. 5.** Effect of UCP2 knockdown on static GSIS by INS-1E pseudoislets. (Panel A) Part of a typical Western blot showing equal presence of UCP2 protein in non-transfected (con, black bar) and scrambled-transfected (scr, white bar) pseudoislets. The bottom band of the shown doublets represents UCP2 as is demonstrated by its relatively low intensity in pseudoislets transfected with *Ucp2*-targeted siRNA (ucp2, grey bar) and its position relative to human recombinant UCP2 protein (rUCP2). UCP2 protein levels were compared between systems as described in detail elsewhere [24] and were normalised to the level detected in non-transfected cells. Bars represent means  $\pm$  SEM of 4 separate pseudoislet preparations. Statistical significance of mean differences was tested by 1-way ANOVA with Dunnett's multiple comparisons posthoc analysis: \*\*\* differs from non-transfected control cells ( $P < 0.001$ ). (Panel B) Normalised insulin secretory response of glucose-starved pseudoislets to 28 mM glucose measured in a static GSIS assay (see Section 2.5). Black, white and grey bars represent means  $\pm$  SEM of 3–4 separate experiments with non-transfected, scrambled-transfected and *Ucp2*-transfected pseudoislets, respectively. Each condition was measured 3 times in all experiments and 2-way ANOVA revealed that differences between means are not statistically significant.



**Fig. 6.** UCP2 knockdown does not affect the temporal kinetics of insulin secretion. Time-resolved insulin secretion was measured as described in "Section 2.6" using INS-1E pseudoislets transfected with scrambled or *Ucp2*-targeted siRNA (white and grey symbols, respectively). Pseudoislets were perfused with buffer containing 200  $\mu$ M glucose and then subjected, sequentially, to 30 mM glucose (G30) and 50 mM KCl at the times indicated by dotted lines. Insulin levels were determined in perfusate fractions taken at 2-min intervals, and insulin secretion was normalised to the average secretory rate observed before glucose addition. Data represent means  $\pm$  SEM of 6 (scrambled-transfected) or 8 (*Ucp2*-transfected) independent experiments. ANOVA for repeated measurements reveals differences between means are not statistically significant.

is not needed to sustain insulin secretion by INS-1E pseudoislets for a prolonged period of time (Fig. 6).

#### 4.1. Molecular function of UCP2

Because of considerable sequence homology with the relatively well characterised UCP1, UCP2 was labelled a *novel* uncoupling protein upon its discovery [27]. However, evidence to confirm that UCP2 indeed does what its label suggests, remains scarce. Direct support for the assertion that UCP2 partially dissipates the mitochondrial protonmotive force, and thus uncouples oxidative phosphorylation, has only been obtained from studies with

reconstituted UCP2 protein or isolated mitochondria [7], i.e., experimental systems of limited physiological relevance. Voza et al. recently revealed that UCP2 catalyses the proton-assisted exchange – across liposome membranes – of phosphate for either malate, oxaloacetate or aspartate [8]. This specific molecular exchange suggests UCP2 exports carbon from the mitochondrial matrix [8], which may attenuate glucose breakdown whilst stimulating glutaminolysis [8], and may thus offer a glucose-sparing metabolic switch [28]. This suggestion agrees with UCP2 effects on glutamine catabolism in macrophages [29] and on glucose/fatty acid oxidation in fibroblasts [30]. The possible fate of UCP2-exported carbon in pancreatic beta cells is less clear, although it is conceivable that UCP2 participates in incompletely understood substrate cycles necessary for glucose catabolism in these cells [31]. A prediction of the carbon export model is that UCP2 limits glucose oxidation [8], but experiments with mouse islets [32] and INS-1E cells [14,15] demonstrate that UCP2 contributes to mitochondrial respiration instead. Consistently, UCP2 knockdown tends to lower basal oxygen uptake (Fig. 3A) and oxygen uptake linked to proton leak (Fig. 3D) in our experiments with serum-deprived BSA control cells grown at high glucose without NEFAs. This contribution of UCP2 to glucose-fuelled respiration is readily explained by uncoupling, but clearer insight in beta cell bioenergetics is needed before other models, perhaps involving carbon substrate cycles, can be excluded.

#### 4.2. UCP2 involvement in glucolipotoxicity

We show here that UCP2 is not involved in palmitate-induced GSIS impairment in INS-1E cells (Fig. 1) and the associated damage to mitochondrial energy metabolism (Fig. 2). These findings are consistent with our observation that palmitate/palmitoleate exposure does not alter UCP2 protein in INS-1E cells [24], but are discrepant with mouse and islet studies reported by others [18,19]. This discrepancy may reflect the limited physiological relevance of INS-1E cells, but as asserted before [15], the relative complexity of

mouse models partly accounts for ongoing debate surrounding the physiological role of UCP2 and its engagement with glucolipotoxicity. Cell models allow UCP2 effects to be probed relatively acutely and under comparably well controlled conditions. Notwithstanding, cells too are sensitive to small differences in experimental design. Coupling efficiency of oxidative phosphorylation is e.g. lowered by palmitate in untreated INS-1E cells [17], but not altered significantly in *siRNA-transfected* cells (Fig. 2B). Also, the protective tendency of palmitoleate against palmitate-induced GSIS impairment (Fig. 1) was not seen before [17].

Mirroring the lack of UCP2 effect on palmitate-induced oxidative phosphorylation defects (Figs. 2 and 3), UCP2 does also not affect the palmitate-provoked formation of mitochondrial superoxide that correlates with INS-1E cell loss [24]. Instead, UCP2 modulates palmitoleate protection against palmitate-related oxidative damage [24]. Monounsaturated NEFAs protect insulinoma cells both against saturated fatty acids and against stress incurred upon cytokine exposure or serum-deprivation [33]. Consistently, UCP2 knockdown protects against the moderate loss of serum-deprived INS-1E cells grown at high glucose [24]. Interestingly in this respect, lipofectamine-mediated transfection of INS-1E pseudoislets with scrambled *siRNA* lowers the absolute rate of insulin secretion, most likely owing to loss of cells – UCP2 knockdown prevents this lipofectamine-induced loss of cells (not shown).

## 5. Conclusion

Our findings disprove the hypotheses we set out to test and we conclude that UCP2 does not mediate palmitate-induced impairment of mitochondrial energy transduction and GSIS in INS-1E cells, and is not necessary for GSIS amplification. Importantly, these ‘negative’ results inform ongoing debate on the enigmatic functions of the beta cell UCP2.

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## Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2015.03.008>.

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