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Charles Affourtit, Martin D Brand. Uncoupling protein-2 contributes significantly to high mitochondrial proton leak in INS-1E insulinoma cells and attenuates glucose-stimulated insulin secretion. *Biochemical Journal*, 2007, 409 (1), pp.199-204. 10.1042/BJ20070954 . hal-00478847

HAL Id: hal-00478847

<https://hal.science/hal-00478847>

Submitted on 30 Apr 2010

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Uncoupling protein-2 contributes significantly to high mitochondrial proton leak in INS-1E insulinoma cells and attenuates glucose-stimulated insulin secretion

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SYNOPSIS

Proton leak exerts stronger control over ATP/ADP in mitochondria from clonal pancreatic beta cells (INS-1E) than in those from rat skeletal muscle, due to the higher proton conductance of INS-1E mitochondria [Affourtit, C. and Brand, M. D. (2006), *Biochem. J.* **393**, 151-159]. Here we demonstrate that high proton leak manifests itself at the cellular level too: the leak rate (measured as myxothiazol-sensitive, oligomycin-resistant respiration) was nearly 4x higher in INS-1E cells than in myoblasts. This relatively high leak activity was decreased more than 30% upon knock-down of uncoupling protein-2 (UCP2) by RNAi. The high contribution of UCP2 to leak suggests that proton conductance through UCP2 accounts for approximately 20% of INS-1E respiration. UCP2 knock-down enhanced glucose-stimulated insulin secretion, consistent with a role for UCP2 in beta cell physiology. We propose that the high mitochondrial proton leak in beta cells is a mechanism to amplify the effect of physiological UCP2 regulators on cytoplasmic ATP/ADP and hence on insulin secretion.

SHORT TITLE

UCP2 and proton leak in INS-1E cells

KEY WORDS

pancreatic beta cells, efficiency of oxidative phosphorylation, mitochondrial uncoupling, UCP2, glucose-stimulated insulin secretion, type 2 diabetes

¹ABBREVIATIONS

FCS, foetal calf serum; GSIS, glucose-stimulated insulin secretion; $\Delta\psi$, mitochondrial membrane potential; UCP, uncoupling protein

INTRODUCTION

Type 2 diabetes is rapidly becoming a global pandemic. Although the development of this metabolic disorder is understood incompletely, it is evident that pancreatic beta cell dysfunction is responsible for its final progression to hyperglycemia. To appreciate fully how type 2 diabetes arises, it is essential to obtain a detailed understanding of beta cell function and regulation. When blood glucose levels rise, beta cells increase their oxidative glucose catabolism, leading to increased mitochondrial protonmotive force and an increased cytoplasmic ATP/ADP ratio. This closes plasma membrane K_{ATP} channels, causing plasma membrane depolarisation, opening of voltage-sensitive calcium channels, calcium influx, and insulin secretion [1]. This canonical model of glucose-stimulated insulin secretion (GSIS¹) relies heavily on efficient mitochondrial energy transduction, since it appears essential that glucose oxidation is coupled tightly to ATP synthesis.

In general, oxidative phosphorylation is coupled incompletely due to proton leakage across the mitochondrial inner membrane. Proton leak in isolated mitochondria is mainly constitutive and depends on the amount of the adenine nucleotide translocase [2]. Importantly, certain proton leak activity is inducible, which provides a mechanism to regulate the efficiency of energy transduction. Inducible proton leak is mostly accounted for by uncoupling proteins (UCPs). The archetypal uncoupling protein, UCP1, catalyses mitochondrial proton conductance in mammalian brown adipose tissue, where it plays the central role in adaptive thermogenesis. The UCP1 orthologues UCP2 and UCP3 mediate mitochondrial proton leak too, but only when activated by (e.g.) superoxide or reactive oxygen species derivatives [3]. The physiological functions of UCP2 and UCP3 remain uncertain [4] and are subject to ongoing debate [5].

Mitochondria from cultured beta cells exhibit GDP-sensitive proton conductance that is stimulated by superoxide [6]. Since UCP2 mRNA is expressed in pancreatic islets, this suggests strongly that beta cells contain a functional UCP2 that is activated by reactive oxygen species [4]. Given its proton-conducting function, it is thought that UCP2 activity attenuates the response of the protonmotive force and ATP/ADP to glucose, and thus impairs GSIS. The most persuasive support for this notion comes from genetic knock-out studies, which reveal that pancreatic islets from *Ucp2*-ablated mice exhibit higher GSIS rates than wild-type controls [7]. Moreover, insulin secretion is improved when endogenous superoxide is lowered using a superoxide dismutase mimetic. Importantly, this effect is observed exclusively in wild-type animals, which demonstrates that UCP2 diminishes GSIS when activated by superoxide [8]. The ability of UCP2 to modulate insulin release is not undisputed: beta cell-specific over-expression of human UCP2 in mice and doxycycline-inducible over-expression of the protein in cultured beta cells did not appear to affect GSIS [9].

In many cell types, e.g. hepatocytes [10], thymocytes [11] and neuronal cells [12], a significant proportion (20-25%) of cellular respiratory activity is used to drive 'futile' mitochondrial proton leak. Despite the potentially pathological effect of UCP2 activity and uncoupling in pancreatic

beta cells, little is known about the magnitude and importance of proton leak in this cell type. We recently predicted that proton leak activity may be relatively high in beta cells, because proton conductance is considerably higher in mitochondria isolated from a well-established clonal beta cell line (INS-1E, see reference [13]) than in those isolated from rat skeletal muscle. As a consequence of its high rate, proton leak exerts a relatively high control over the membrane potential ($\Delta\psi$) and ATP/ADP ratio in INS-1E mitochondria [14]. The nature of this relatively high proton conductance is unclear, however, and it is uncertain whether the high leak in INS-1E mitochondria is of physiological relevance or merely represents a mitochondrial isolation artefact.

In the present study we show that, similar to the situation in isolated mitochondria, proton leak rates are significantly higher in INS-1E cells than in myoblasts. Myxothiazol-sensitive, oligomycin-resistant respiratory activity (a good approximation of leak) is roughly 4-fold higher in INS-1E than in muscle cells. RNA interference (RNAi) experiments demonstrate that over 30% of the leak activity in INS-1E cells is accounted for by UCP2. We estimate that roughly 20% of INS-1E respiration is caused by proton cycling through UCP2. In agreement with the loss of function studies in mice, GSIS is enhanced upon UCP2 knock-down in INS-1E cells.

EXPERIMENTAL

Cell culture and mitochondrial isolation

INS-1E cells were grown as described in [13] in medium containing 11 mM glucose; antibiotics were omitted in RNAi experiments. C2C12 myoblasts were grown at 37°C under 5% CO₂ in Dulbecco's Modified Eagle's medium (Sigma-Aldrich, Gillingham, UK) supplemented with 10% (v/v) foetal calf serum (FCS) and 4 mM glutamine. Mitochondria were prepared as described in [14]. INS-1E cells and myoblasts harvested from 500 cm² trays (Nunclon; 80-90% confluence) yielded typically ~10 and 3 mg mitochondrial protein per tray, respectively. The comparatively low yield from myoblasts is due to the relatively high mechanical force required to rupture cells of this type. Importantly, mitochondria from INS-1E cells and myoblasts were well coupled, with respiratory control ratios during succinate oxidation of ~3 and 4, respectively.

UCP2 knock-down and detection

siRNA oligos (Ambion, Huntingdon, UK) were targeted at rat *Ucp2* exons 3, 5 and 8 (Table 1). Their ability to lower UCP2 levels was assessed in cells, seeded in 175 cm² flasks (BD Falcon™), grown to ~50% confluence. Following complex-formation, siRNA and the transfection agent Lipofectamine™ 2000 (Invitrogen, Paisley, UK) were added at final concentrations of 50 nM and 1.7 µg/mL, respectively. To identify potential non-specific effects, cells were transfected in parallel with scrambled siRNA (Ambion, *Silencer*® Negative Control 1). After 3 days' further growth, transfected cells were trypsinized; mitochondria were then isolated, resuspended and stored at -80°C in gel-

loading buffer (10% (w/v) SDS, 250 mM Tris-HCl (pH 6.8), 5 mM EDTA, 50% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 0.05% (w/v) bromophenol blue). To detect UCP2, 20 μ g mitochondrial protein was separated on a 12% SDS polyacrylamide gel and transferred to nitrocellulose. The membrane was blocked for 2 h at room temperature in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) dried skimmed milk (Marvel), then probed overnight at 4°C with goat anti-UCP2 1° antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 0.2 μ g/mL in this blocking buffer. Subsequently, the membrane was incubated for 1 h at room temperature with peroxidase-conjugated 2° antibodies (Pierce Biotechnology, Rockford, IL, USA) diluted to 0.1 μ g/mL in blocking buffer, then cross-reacted proteins were visualised by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK). Finally, the membrane was stained with GelCode® Blue reagent (Pierce Biotechnology, Rockford, IL, USA) to confirm equal protein loading. Protein was quantified by densitometry using *ImageJ* software (<http://rsb.info.nih.gov/ij/>).

Mitochondrial proton leak

O₂-uptake rates and $\Delta\psi$ were measured simultaneously as described in [14]. Mitochondria were incubated at ~0.9 mg/mL with 350 ng/mL nigericin to collapse Δ pH, 4 μ M rotenone to inhibit complex I, and 0.9 nmol oligomycin/mg protein to inhibit phosphorylation. Succinate (4 mM) was added to initiate state 4 respiration, which was titrated with malonate (up to 0.4 mM) causing stepwise inhibition of respiratory chain activity and decrease of $\Delta\psi$. Expression of the successive O₂-uptake rates as a function of the concomitant $\Delta\psi$ values revealed the kinetic response of proton leak to $\Delta\psi$.

Cell respiration

Non-transfected INS-1E cells and myoblasts, seeded in 175 cm² flasks, were grown to ~80% confluence. In RNAi experiments, cells were grown to roughly 50% confluence, transfected as described above, and then grown another 3 days. Cells were then trypsinized, resuspended in their respective growth media at roughly 10⁷ cells/mL, and stored on ice. Cell aliquots (600 μ L) were put in glass vessels, flushed with 5% CO₂-95% air, sealed, incubated for 30 min in a shaking (100 cycles/min) water bath at 37°C, and then transferred to an oxygraph. Respiratory rates were calculated from the initial steady parts of the O₂-uptake traces and expressed per 10⁶ viable cells. Experiments were also performed with 7 μ M oligomycin to approximate proton leak, and 9 μ M myxothiazol to determine non-mitochondrial O₂-uptake. Inhibitors were dissolved in DMSO (final concentration \leq 0.2%) and added just before flushing with CO₂-air. Both concentrations gave maximum effects as checked by doubling concentrations.

Insulin secretion

Cells were seeded in 24-well plates (BD Falcon™) at 3×10^5 cells/well, grown overnight, and then transfected with UCP2-targeted (exon 8) or scrambled siRNA. After 3 days' further growth, transfected cells were starved for 2 h in growth medium that lacked glucose and pyruvate, and contained only 1% (v/v) FCS. Cells were washed 2x with a glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH: 135 mM NaCl, 3.6 mM KCl, 10 mM Hepes (pH 7.4), 0.5 mM MgCl₂, 1.5 mM CaCl₂, 5 mM NaHCO₃, 0.5 mM NaH₂PO₄ and 0.1% (w/v) bovine serum albumin) and were then incubated in this buffer for 30 min in a shaking (30 cycles/min) water bath at 37°C. Next, medium was replaced with KRBH containing 2, 5 or 30 mM glucose, or 2 mM glucose + 30 mM KCl. After a further 30 min, medium was collected and centrifuged to pellet any detached cells. Supernatants were assayed for insulin by ELISA (Mercodia, Uppsala, Sweden) using mouse insulin as a standard.

Statistics

Mean differences between experimental systems and conditions were tested for statistical significance by Student's *t*-tests.

RESULTS

Higher proton leak in INS-1E cells than myoblasts

INS-1E mitochondria exhibit proton conductance that is significantly higher than that observed in mitochondria from rat skeletal muscle [14]. However, it may be that this difference solely indicates a discrepancy between cell and tissue mitochondria, or merely reflects a mitochondrial isolation artefact. As evidenced by the data shown in Fig. 1, both these concerns are ungrounded. Fig. 1A demonstrates that proton leak rate in myoblast mitochondria is substantially lower at any measured $\Delta\psi$ than the equivalent rate in INS-1E mitochondria. The considerable leak difference between mitochondria also manifests itself when leak is measured in intact cells. The proton leak rate in cells was estimated as the intracellular mitochondrial respiration rate (defined as respiration sensitive to the electron transport chain inhibitor myxothiazol) that was not sensitive to the ATP synthase inhibitor oligomycin. This method overestimates the leak rate a little because oligomycin causes a small increase in $\Delta\psi$ that slightly increases leak, but based on experiments with hepatocytes [10] the overestimate is not expected to be large. Although total INS-1E O₂-uptake is only about half of that observed in myoblasts, the absolute oligomycin-resistant rate is nearly 4x higher in INS-1E cells, and relative to the rate without oligomycin, the rate with oligomycin is 7x higher in INS-1E cells (Fig. 1B). These cellular differences are higher than predicted from the mitochondrial data, which illustrates the relative importance of respiratory experiments in intact cells.

From Fig. 1B, the ratio of O₂-uptake rates with and without oligomycin can be calculated.

This ratio provides an estimation of the proportion of respiratory activity that is used to drive 'futile'

mitochondrial proton leak in INS-1E (0.76 ± 0.06) and muscle cells (0.11 ± 0.02). Thus 75% of respiration would appear to drive proton leak in INS-1E cells, whilst only 10% of respiration in myoblasts is used for this purpose. Keeping in mind that the *in vitro* total respiratory rates used to calculate these proportions are not necessarily representative of those prevalent *in vivo*, the obtained numbers may be compared with similar ratios reported in the literature. The equivalent values are ~25% in hepatocytes [10], and ~20% in thymocytes [11] and neuronal cells [12]. The high INS-1E value disagrees with a value of 10% in INS-1 cells [9], perhaps due to extensive differences in experimental conditions. Alternatively, this discrepancy could be related to the different origins of the respective cell lines. INS-1 is an insulin-secreting line that was isolated from a radiation-induced rat insulinoma [15]. Because of their non-clonal nature, INS-1 cells exhibit a limited stability over passages, which has been suggested as a possible explanation for the large variability in published results [13]. INS-1E on the other hand, is a clonal line derived from the parental INS-1 cells [16], has been characterised in detail, and represents a significant improvement in terms of beta cell differentiation and stability [13].

UCP2 contributes to high INS-1E proton leak

Given the pronounced effect of UCP2-ablation on GSIS in pancreatic islets [7], we reasoned that the relatively high proton leak activity observed in INS-1E cells (Fig. 1B) could be due to UCP2 activity. We used RNAi to reduce UCP2 levels, and thus test this hypothesis. We routinely use a polyclonal antibody to detect UCP2 in mouse tissues, and find no UCP2 band in samples from UCP2 knockout mice (not shown). Fig. 2A shows that in INS-1E cells this polyclonal antibody cross-reacts with a single protein of ~32 kD in samples obtained from both non-transfected cells (lane 1) and those transfected with scrambled siRNA (lane 5). The intensity of this band is reduced dramatically in samples from cells that have been transfected, separately, with three different siRNA oligonucleotides targeted at independent *Ucp2* exons (lanes 2-4). Densitometric comparison confirms equal loading of total mitochondrial protein (Figs 2B and 2D), and reveals that UCP2 levels are reduced substantially within 72 h after transfection (Fig. 2C). The observed densities upon UCP2 knock-down are roughly 15% (exons 5 and 3) and 10% (exon 8) of the scrambled control.

O₂-consumption rates of cells transfected with scrambled siRNA were 10.6 ± 2.1 in the absence and 7.7 ± 1.5 nmol O/min/10⁶ cells in the presence of oligomycin. These activities are comparable with those observed in non-transfected INS-1E cells (Fig. 1A). The overall respiratory activity of cells transfected with *Ucp2*-targeted siRNA is on average nearly 13% lower than that of cells transfected with scrambled siRNA (Fig. 3A). This difference is statistically significant ($p = 0.02$) and, importantly, it arises irrespective of the particular *Ucp2* exon targeted. Similarly, oligomycin-insensitive respiratory activity is lowered, on average by more than 30% ($p = 1 \times 10^{-5}$), when UCP2 levels are diminished by siRNA targeted at 3 separate exons (Fig. 3B). As a result, the fraction of overall respiration that is oligomycin-resistant is reduced significantly ($p = 0.01$) upon UCP2 knock-

down (Fig. 3C). This suggests strongly that UCP2 contributes substantially to the proton leak rate of non-transfected INS-1E cells.

In this context, it is anticipated that $\Delta\psi$ increases upon UCP2 knock-down. This will not only lead to an underestimation of UCP2's contribution to proton leak (see Discussion), but may also result in increased reactive oxygen species levels. It is possible that this in turn activates additional uncoupling mechanisms. However, the nature of UCP2-independent proton leak is unclear at present.

UCP2 knock-down enhances insulin secretion

In the current experiments, insulin secretion rates in INS-1E cells are relatively high at 2 mM glucose. However, these rates are increased significantly when the glucose level is raised to 30 mM, or cells are incubated in the additional presence of 30 mM KCl (Fig. 4A). Since INS-1E cells are expected to respond to such stimuli in this manner [13], we deemed our assay suitable to assess the effect of UCP2 knock-down on GSIS. In cells transfected with scrambled siRNA, insulin secretion is similar at 2 and 5 mM glucose (Fig. 4A), but whilst UCP2 knock-down does not affect secretion at 2 mM glucose, it enhances insulin release at 5 mM glucose. This indicates GSIS is improved, which agrees with observations made in islets from *Ucp2* knock-out mice [7]. Upon UCP2 knock-down, insulin secretion is also increased at 30 mM glucose but, importantly, KCl-induced secretion, which does not rely on mitochondrial energy metabolism, is not affected (Fig. 4A). From Fig. 4B it can be seen that cell number and cellular insulin content are not altered significantly after UCP2 down-regulation, although the average total insulin level appears somewhat increased.

DISCUSSION

Because the cytoplasmic ATP/ADP of pancreatic cells is governed predominantly by ATP supply [1], these cells are interesting from a bioenergetic perspective. Glucose-induced increases in ATP/ADP observed in cells [1] are not seen in most other cell types, where this ratio is mainly controlled by ATP demand [17]. Based on a metabolic control analysis of oxidative phosphorylation in muscle and INS-1E mitochondria, we suggested recently that this difference in control is partly due to mitochondrial peculiarities [14]. In particular, proton leak rate was relatively high in INS-1E mitochondria and, consequently, exerted relatively high control over $\Delta\psi$ and the external ATP/ADP ratio. The data reported here demonstrate that a relatively high proton conductance does not merely represent a mitochondrial isolation artefact, but is a characteristic feature of intact INS-1E cells (Fig. 1B). The estimated proportion of overall respiratory activity used to drive 'futile' proton leak in INS-1E cells is much larger than that used in hepatocytes [10], thymocytes [11] or neuronal cells [12].

Our results establish that a large part of the high leak rate in INS-1E cells is accounted for by UCP2, as oligomycin-resistant O_2 -consumption is decreased by ~30% when the protein is knocked down using RNAi (Fig. 3B). Fig. 3A indicates that UCP2 also contributes to the total respiratory

activity. A contribution of 13% is an underestimate, however, because UCP2 knock-down will raise $\Delta\psi$ and cause a compensatory increase in proton leak. A better estimate comes from multiplying the slightly underestimated contribution of UCP2 to the oligomycin-insensitive respiration (30%) by the slightly overestimated proportion of overall respiration that drives proton leak (75%) to give a value of about 20% of the cell respiration rate caused by proton cycling through UCP2 in cells. This value is calculated assuming RNAi removes all UCP2, which will cause a further small underestimation of its contribution. Nonetheless, 20% is high compared to the calculated 10% in thymocytes: ~20% of thymocyte respiration is caused by proton cycling [11] and ~50% of this is lost in thymocytes from UCP2 knock-out mice [18].

The relatively high contribution of UCP2 to INS-1E respiration (Fig. 3), its attenuating effect on GSIS (Fig. 4A), and the significant control of proton leak over ATP/ADP [14] support the view that UCP2 may play an important regulatory role in cell physiology. Our data indicate that UCP2 is most likely active under pathological *and* physiological conditions with its activation state being dependent on fluctuating levels of effectors such as superoxide [6]. In other words, UCP2 activation is unlikely just a pathological side effect of a damage limitation mechanism that is induced during high-fat feeding or hyperglycemia and that causes cell dysfunction [7, 8, 19]. We suggest that the relatively high mitochondrial proton leak in cells is a mechanism that amplifies the effect of physiological regulators of UCP2 proton conductance on the cytoplasmic ATP/ADP ratio and hence on GSIS.

The notion that UCP2 regulates GSIS is corroborated by a steadily increasing body of evidence. Initial studies showed that adenoviral over-expression of UCP2 leads to attenuated GSIS in rats [20] and insulinoma cells [21]. Despite the fact that over-expression of UCPs tends to cause non-specific proton conductance [3], such observations demonstrate that GSIS is impaired when oxidative phosphorylation becomes less efficient. More importantly, investigations involving *Ucp2*-ablated mice showed convincingly that UCP2 is a negative modulator of GSIS under conditions that are of (patho)physiological relevance [7, 8, 19, 22]. Our results (Fig. 4A) agree with these gene knockout studies: lowering UCP2 levels in INS-1E cells through RNAi results in increased GSIS. INS-1E data are inevitably of less physiological relevance than those obtained using islets. Arguably, however, the interpretation of experiments with *Ucp2*-knockout mice is relatively complicated, since the *ab initio* absence of the *Ucp2* gene may have caused potentially complex developmental adaptations. Additionally, the observed altered islet behaviour may be an indirect result from the absence of *Ucp2* in other tissues such as the brain. Because our experimental model does not suffer from such complications, the data reported in this paper demonstrate that GSIS can be modulated *acutely* by UCP2 activity. Such acute regulation is in line with observed immediate effects on GSIS of a superoxide dismutase mimetic [8] and a pharmacological UCP2 inhibitor [22]. It is also worth notice that acute short-term inhibition of UCP2 expression by antisense oligonucleotides has recently been shown to ameliorate the hyperglycemic syndrome in two different animal models of type 2 diabetes

[23]. Interestingly, this metabolic improvement appears to be due to a combined effect of UCP2 knock-down on insulin secretion by pancreatic cells and insulin action on peripheral tissues.

Despite the mounting evidence for a GSIS-modulatory role, it remains uncertain whether UCP2 indeed affects insulin secretion through uncoupling ATP synthesis from mitochondrial electron transfer. Previously reported effects of UCP2 on ATP/ADP (e.g. [7, 23]), $\Delta\psi$ [8, 22], and O_2 -consumption [24] favour such an uncoupling function, but are indirect measures providing circumstantial support only. In fact, the strongest direct indication that UCP2 uncouples oxidative phosphorylation *in situ* in any mammalian cell, comes from kinetic experiments with thymocytes [18]. At any given $\Delta\psi$, thymocytes from *Ucp2* knock-out mice exhibit lower non-phosphorylating (i.e. state 4) respiratory activity than wild-type cells. The effect of UCP2 knock-down on oligomycin-resistant (i.e. state 4) O_2 -uptake in INS-1E cells (Fig. 3) is in close agreement with the kinetic measurements in thymocytes, and provides direct evidence for an uncoupling function of UCP2 in cells.

The results reported in this paper validate predictions of relatively high proton leak activity in INS-1E cells, and demonstrate that UCP2 contributes substantially to this activity. They show furthermore that UCP2 modulates insulin secretion in INS-1E cells in a fashion that is similar, qualitatively, to that observed in pancreatic islets. Our work thus highlights that INS-1E cells are a convenient and appropriate model to further clarify the role of UCP2 in islet physiology.

ACKNOWLEDGEMENTS

INS-1E cells were kindly donated by Drs P. Maechler and C. Wollheim (Department of Internal Medicine, University Medical Center, Geneva, Switzerland). This work was supported by the Medical Research Council, United Kingdom.

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Table 1 – siRNA oligonucleotide sequences (5'→3' sense) targeted at the rat *Ucp2*

exon 3	GAUCUCAUCACUUUCCCUCtt
exon 5	GCACUGUCGAAGCCUACAAtt
exon 8	CGUAGUAAUGUUUGUCACtt

Stage 2(a) POST-PRINT

FIGURE LEGENDS

Figure 1 – Higher proton leak in INS-1E cells than myoblasts

A: Proton leak (J_i) dependence on $\Delta\psi$ was measured in INS-1E (●) and myoblast (○) mitochondria. Data and error bars represent mean \pm SEM of 5 (●) and 6 (○) samples; data were fitted to exponentials. *B*: Whole-cell respiration (J_o) \pm 7 μ M oligomycin was measured in myoblasts (open) and INS-1E cells (grey); non-mitochondrial activity (3.3 ± 0.4 and 2.8 ± 0.3 nmol O/min/ 10^6 myoblasts and INS-1E cells, respectively) was subtracted. Data and error bars represent means \pm SEM of 4 experiments.

Figure 2 – UCP2 knock-down

A: Western blot (UCP2) of mitochondrial protein from non-transfected INS-1E cells (1) and cells transfected with scrambled siRNA (5) or siRNA targeted at *Ucp2* exons 5, 8, and 3 (2-4, respectively). *B*: nitrocellulose membrane stained with GelCode® Blue after Western analysis; contrast was enhanced *in silico* to aid visualisation. *C*: Densitometric profiles of lanes 2-5 of Western blot shown in panel *A*. *D*: Whole-lane relative densitometric quantification of protein stained with GelCode® Blue; quantification was performed using an image of which the contrast had not been enhanced. Numbers left to panel *A* represent molecular weight markers (kDa).

Figure 3 – UCP2 knock-down lowers proton leak

Respiration (J_o) was measured in the absence (*A*) or presence (*B*) of 7 μ M oligomycin in INS-1E cells transfected with scrambled siRNA (black), or siRNA targeted at UCP2 exons 5 (dark grey), 8 (light grey) or 3 (open); non-mitochondrial activity (3.0 ± 0.7 , 2.0 ± 0.5 , 1.9 ± 0.4 and 1.7 ± 0.5 nmol O/min/ 10^6 cells, respectively) was subtracted. Data and error bars represent means \pm SEM of 5 experiments and are expressed as a fraction of the scrambled control rates (*A* and *B*), or as the oligomycin-insensitive respiratory fraction (*C*). Scrambled control rates \pm oligomycin were 7.7 ± 1.5 and 10.6 ± 2.1 nmol O/min/ 10^6 cells, respectively.

Figure 4 – UCP2 knock-down enhances GSIS

INS-1E cells were transfected with scrambled (grey) or UCP2-exon 8 (open) siRNA. *A*: Insulin secretion was measured at 2, 5 and 30 mM glucose (G2, G5, G30), and at 2 mM glucose + 30 mM KCl (KCl). Data and error bars represent means \pm SEM of 6 (G2, G5, G30) and 12 (KCl) experiments with each condition assayed 4-8x. *B*: Cells were counted on a haemocytometer after trypsinization from 24-well plates (3 wells were pooled for each count). Total cellular insulin content was measured in acidified-ethanol extracts. Data and error bars represent means \pm SEM of 3 experiments performed in quadruplicate.







