








## RESEARCH ARTICLE

**REVISED** **Insights into pancreatic  $\beta$  cell energy metabolism using rodent  $\beta$  cell models [version 3; peer review: 2 approved, 1 not approved]**

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### Abstract

**Background:** Mitochondrial diabetes is primarily caused by  $\beta$ -cell failure, a cell type whose unique properties are important in pathogenesis.

**Methods:** By reducing glucose, we induced energetic stress in two rodent  $\beta$ -cell models to assess effects on cellular function.

**Results:** Culturing rat insulin-secreting INS-1 cells in low glucose conditions caused a rapid reduction in whole cell respiration, associated with elevated mitochondrial reactive oxygen species production, and an altered glucose-stimulated insulin secretion profile. Prolonged exposure to reduced glucose directly impaired mitochondrial function and reduced autophagy.







**Conclusions:** Insulinoma cell lines have a very different bioenergetic profile to many other cell lines and provide a useful model of mechanisms affecting  $\beta$ -cell mitochondrial function.

### Keywords

beta-cell, oxidative phosphorylation, reactive oxygen species, superoxide, mitochondria, insulin secretion

### Open Peer Review

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**Competing interests:** JH is an employee of Luxcel Biosciences. None of the other authors have competing interests.

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**REVISED Amendments from Version 2**

We have reviewed our statistical analysis and revised the figures accordingly including revision of figures to improve clarity. The main message of the paper remains the same.

**Any further responses from the reviewers can be found at the end of the article**

**Abbreviations**

Reactive oxygen species (ROS), uncoupling protein 2 (UCP2), oxidative phosphorylation (OXPHOS), photomultiplier tubes (PMT), isolation buffer (ISO), respiratory control ratio (RCR), 2'-7'-dichlorofluorescein diacetate (H<sub>2</sub>DCFDA,SE), Glucose stimulated insulin secretion (GSIS), endoplasmic reticulum (ER).

**Introduction**

Mitochondria play a key role in glucose homeostasis<sup>1,2</sup>. Maternally inherited diabetes is caused by a mtDNA mutation with a population prevalence of 1 in 300<sup>3</sup>, affecting up to 1% of patients with diabetes<sup>4,5</sup> and often going unrecognized by clinicians. Mitochondrial dysfunction in highly metabolically active pancreatic islets results in abnormal  $\beta$ -cell function linked to insulin deficiency (reviewed in 6). In patients whose diabetes is associated with mitochondrial DNA mutations, reduced  $\beta$ -cell mass may result from islet hypoplasia<sup>7</sup> or apoptosis<sup>8</sup>, although the latter has never been demonstrated *in vivo* or *post mortem* tissue<sup>9</sup>. The limited *post mortem* studies suggest that the mutant load of the pathogenic 3243 A-G mtDNA mutation in t-RNA leucine is low in the pancreas compared to other affected tissues, 30% contrasting with >70% in other tissues<sup>10</sup>. This observation suggests that beta cells with high mutant load are likely to fail, and this is linked to reduced  $\beta$ -cell mass. In other affected tissues in patients carrying mtDNA mutations, mutant load is clearly linked to mitochondrial dysfunction<sup>11</sup>.

The lack of a human beta cell model has made studying mitochondrial associated  $\beta$ -cell dysfunction *in vitro* challenging. Much of the relevant data comes from models based on other cell types, including cancer cell derived hybrids<sup>12</sup> and more recently neurons derived from induced pluripotent stem cells (IPS) cells<sup>13</sup>. The specialized nature of the pancreatic  $\beta$ -cell, as a glucose sensor, supported by multiple specialized pathways of intermediary metabolism, makes comparison with other cell models particularly problematic. In the  $\beta$ -cell, mitochondria appear pivotal in regulating glucose stimulated insulin secretion (GSIS). Increasing glucose uptake not only increases ATP generation, but also activates mitochondrial metabolic pathways<sup>14</sup>. Although metabolism and mitochondrial function play a role in driving insulin secretion under raised glucose conditions, it remains unclear which of the cellular signaling pathways involved is the key driver of the process. Reactive oxygen species (ROS) are not always deleterious, but are essential components of biological processes<sup>15,16</sup>. For instance, plasma membrane K<sub>ATP</sub> channels can be inhibited either by elevated levels of ATP<sup>17</sup> or by raised levels of mitochondrial

ROS without raised ATP levels<sup>18</sup>. Both processes are able to drive insulin secretion via changes in cellular calcium influx.

The pancreatic  $\beta$ -cell mitochondria are compact and constantly involved in fusion and fission activity, which is altered depending upon nutrient exposure<sup>19</sup>. Mitochondrial membrane potential within the mitochondrial network also varies with substrate exposure, becoming more heterogeneous following a low glucose or high glucose plus lipid challenge<sup>20</sup>. Autophagy is essential for  $\beta$ -cell quality control; specific disruption of the autophagy process results in swollen mitochondria, high levels of ubiquitination and distention of the endoplasmic reticulum (ER)<sup>21,22</sup>. In rat insulin-secreting INS-1 cells, autophagy has been shown to be involved in the removal of dysfunctional mitochondria with low mitochondrial membrane potential following mitochondrial fission<sup>23</sup>. Work by Affourtit *et al.*<sup>24</sup>, showed that the  $\beta$ -cell appears to have "metabolic safety valves" in place. For example, the mitochondrial uncoupling protein UCP2 seems to prevent over-stimulation of oxidative phosphorylation (OXPHOS) and excessive ROS production<sup>25</sup> during periods of high metabolic activity and insulin secretion. Hence, predicting how mitochondrial dysfunction associated with a mutation or substrate supply will impact on a  $\beta$ -cell is difficult. Common variants of mtDNA associated with type 2 diabetes have been identified and increase disease susceptibility in a wide range of populations<sup>26</sup>. Studying the functional effects of these variants in a human  $\beta$ -cell model is not currently possible. We therefore tested rodent insulinoma models to determine whether they are suitable for studying the effects of mitochondrial dysfunction *in vitro*. An ideal *in vitro*  $\beta$ -cell model for studying mitochondrial dysfunction should demonstrate that GSIS is strongly linked to increased mitochondrial respiration as glucose levels are raised, linking increase energy metabolism to insulin secretion. Models that use  $\beta$ -cells derived from IPS cells are not yet robust. In this study we use two rodent  $\beta$ -cell lines INS-1 and MIN-6 to investigate the effect of glucose exposure on cell viability, mitochondrial function, GSIS and autophagy.

**Methods****Chemicals**

Unless otherwise stated, all chemicals used in this study were obtained from Sigma, UK.

**Cell lines**

INS-1 cells were cultured in RPMI 1640 media (11mM glucose; Invitrogen), containing 15% fetal calf serum, 25mM HEPES, 50 $\mu$ M  $\beta$ -mercaptoethanol, 2mM L-glutamine, 100 $\mu$ g/ml streptomycin and 100U/ml penicillin. MIN-6 cells were cultured in DMEM media (25mM glucose; Invitrogen), containing 15% fetal calf serum, 1mM pyruvate, L-Glutamax, 50 $\mu$ M  $\beta$ -mercaptoethanol, 100 $\mu$ g/ml streptomycin and 100U/ml penicillin. Both cell lines were maintained under normoxic conditions in air plus 5% CO<sub>2</sub> at 37°C. The data presented for both cell lines was accumulated from cultures between passages P22-P40. Glucose free media used in subsequent experiments for the two lines was identical to the standard culture media, except for

the glucose free DMEM, which did not contain pyruvate. Glucose was then added at different concentrations depending upon the experiment. The MIN-6 and INS-1 cells were provided by Professor Patrik Rorsman (OCDEM, University of Oxford). MIN-6 and INS-1 cells were initially derived by Professors Miyazaki and Wolheim.

We also used HepG2 and U87MG cells (obtained from ATCC) for comparison as they show a reduction in mitochondrial respiration as glucose levels are raised<sup>27</sup>. HepG2 and U87MG lines were cultured in DMEM media (25mM glucose), containing 10% fetal calf serum, 1mM pyruvate, 2mM L-glutamine, 100µg/ml streptomycin and 100 U/ml penicillin. For whole cell respiration and flow cytometry assays, which require cells to be in suspension, we used Accutase (PAA), a formulated mixture of digestive enzymes, to release cells from the tissue culture flasks. Several studies have shown Accutase to be less damaging to cells than trypsin<sup>28,29</sup>.

#### Glucose stimulated insulin secretion (GSIS) assay

Initial experiments to demonstrate that INS-1 and MIN-6 cells show a GSIS response were carried out by culturing cells in 6 well plates. To assess the impact of a one hour reduced glucose challenge on the GSIS response of INS-1 cells, experiments were carried out as follows; INS-1 cells (100,000 per well) were cultured for 4 days in 12 well plates (Nunc). Prior to carrying out the GSIS assay, one of the plates was pre-incubated in RPMI 1640 media containing 1mM glucose for one hour. Media was then removed from both plates and cells were washed in pre-warmed Krebs Ringer Buffer (modified KRB: 2.54mM CaCl<sub>2</sub>, 1.199mM KH<sub>2</sub>PO<sub>4</sub>, 4.64mM KCL, 25mM NaHCO<sub>3</sub>, 1.19mM MgSO<sub>4</sub>, 10mM HEPES [pH 7.4]). 2ml of pre-warmed KRB containing 1, 5, 10 or 15mM glucose was added and incubated for 30 minutes. Each well was aspirated and replaced with 2ml of KRB containing the same concentrations of glucose and incubated for another 30 minutes. The supernatant was collected and assayed for insulin using a mouse insulin ELISA (Merckodia; catalog no., 10-1247-01-10). GSIS insulin secretion experiments were also carried out without the one hour pre-incubation in RPMI 1640 containing 1mM glucose.

#### Assessment of cell growth

Cell growth/number under different growth conditions was assessed by determining cell number using a haemocytometer.

#### Media glucose and lactate levels

Glucose was measured using the glucose oxidase, and lactate by the lactate oxidase assay on a Siemens ADVIA 2400 chemistry analyser. These are standard test carried out in NHS labs based on the hexokinase method (Glucose) and a method for lactate<sup>30,31</sup>.

#### Mitochondrial and cellular ROS measurements

*MitoSox and flow cytometry:* MitoSox red (Invitrogen), a derivative of dihydroethidium, which is taken up by mitochondria in whole cells, was used to measure cellular mitochondrial ROS levels<sup>32,33</sup>. INS-1 and MIN-6 cells were grown in 175 cm<sup>2</sup> flasks under standard tissue culture conditions. Cells were harvested using Accutase and washed with PBS.

*Determination of non-saturating doses of MitoSox:* The MitoSox measurement of mitochondrial ROS relies upon the formation of the MitoSox/ROS product binding to mtDNA to produce mitochondrial fluorescence. Previous studies have shown that MitoSox labeling doses must be carefully titrated to avoid saturation of the dye (i.e. fluorescence rapidly reaches a maximum at high dye concentrations due to saturation of all of the available mtDNA)<sup>32</sup>. As mtDNA levels are likely to be limiting in this reaction, we initially determined the concentration of MitoSox, which showed a linear increase in mitochondrial superoxide over a 3–4 hour period. In total, 100,000 cells for each cell line were incubated with 10nM, 100nM, 500nM, 1µM and 5µM MitoSox in DMEM (25mM glucose)/RPMI (11mM glucose) for 10 minutes. Cells were pelleted and washed twice in PBS before incubation in DMEM/RPMI containing 0mM glucose. Previous experiments had shown that 0mM glucose generated the highest amount of mitochondrial ROS compared to higher glucose incubations. As shown in [Supplementary Figure 1](#), both cell lines show a non-saturating mitochondrial ROS production when cells are incubated for 10 minutes in media containing 5µM MitoSox prior to exposure to low glucose. Exposure to lower doses of MitoSox for 10 minutes did not detect significant levels of mitochondrial ROS in this system.

For glucose experiments, MIN-6 and INS-1 cells were grown under standard tissue culture conditions until 80% confluent. Cells were harvested and incubated with 5µM MitoSox for 10 minutes in standard tissue culture conditions. Following MitoSox staining, cells were washed with PBS and then split into six aliquots and incubated with different concentrations of glucose (0mM, 1mM, 2.5mM, 5mM, 10mM and 20mM). Cells were taken from the various treatments for analysis by flow cytometry at different time points. All samples were analyzed using a BD LSRII flow cytometer (BD Biosciences, Oxford, UK). MitoSox Red was excited using a 488nm laser and emission was detected using a bandpass filter of 575/26. Single cells were gated and 10,000 total cell events were collected. Data was analyzed using BD FACS Diva 5.0 software and mean fluorescence intensity (MFI) was compared. Under these conditions both the mitochondrial superoxide specific 2-hydroxyethidium (2-OH-E<sup>+</sup>) and ethidium cation (E<sup>+</sup>) are detected.

*Measuring mitochondrial ROS and cellular peroxide generation during low glucose exposure:* Cells were cultured as above, harvested using Accutase, and re-suspended in 8ml of DMEM (25mM glucose, MIN-6) or RPMI (11mM glucose, INS-1), containing a non-saturating concentration of MitoSox (5µM) and incubated for 10 minutes at 37°C. Additionally, cells were also incubated in standard cell culture media, containing the 2'-7-dichlorofluorescein derivative H2DCFDA,SE (Oxyburst Green) at a concentration of 10µM for 30 minutes at 37°C. Cells were pelleted and washed twice in PBS. Approximately 100,000 cells were then aliquoted into 1.5 ml Eppendorf tubes, pelleted and re-suspended in media containing the various treatment regimens and incubated at 37°C under standard tissue culture conditions (5% CO<sub>2</sub>, 20% O<sub>2</sub>). Holes were made in the lids of each tube to allow media equilibration with oxygen and 5% CO<sub>2</sub>. Following incubation for 60 minutes, cells were pelleted and

re-suspended in PBS containing 0.5% FCS and kept on ice prior to flow cytometric analysis. The Oxyburst Green signal was analyzed using Beckman Coulter Epics Altra flow cytometer. In total, 10,000 events were collected and data was analyzed by Beckman Coulter Expo 32 software Photomultiplier tubes (PMTs), which collect fluorescent light at 525nm. Cell populations were initially identified on the basis of size (forward scatter) versus granularity (side scatter) and gated on these physical characteristics.

*Determination of whole cell superoxide levels using dihydroethidium (DHE) and high performance liquid chromatography (HPLC) analysis:* Measurement of 2-hydroxyethidium (2-OHE<sup>+</sup>) formation by HPLC was used to detect cellular superoxide production, using methods adapted from those described previously<sup>34</sup>. Cells were harvested with Accutase, washed once in PBS and incubated in standard tissue culture media containing DH (25µmol/l) for 20 minutes at 37°C. The cells were pelleted by centrifugation at 1,100xg for 3 minutes, washed twice in PBS and incubated in the dark at 37°C in media containing 1mM, 2.5mM, 5mM, 10mM or 20mM glucose for 3 hours. Cells were then pelleted, washed once in PBS and snap frozen in liquid nitrogen prior to storage at -80°C until required. The cells were lysed in ice-cold methanol. Hydrochloric acid (100mM) was added (1:1 v/v) prior to loading into the HPLC autosampler for analysis. All the samples were protected from light at all times. Separation of DHE, 2-OHE, and ethidium was performed using a gradient HPLC system (Jasco LTD, UK, Model 542) with an ODS3 reverse phase column (250 mm, 4.5 mm; Hichrom) and quantified using a fluorescence detector set at 510 nm (excitation) and 595 nm (emission). All results were subtracted with the negative (non-treated with DHE) control.

#### Whole cell oxygen consumption measurements

*Oxygen electrode:* Whole cell oxygen consumption was determined using a Clark type oxygen electrode (Hansatech). Cells were incubated under the appropriate conditions, detached with Accutase, washed in PBS and re-suspended in glucose free RPMI/DMEM. Respiration rates were determined when cells were respiring at their maximal rates and expressed in nmoles oxygen consumed per minute.

*MitoXpress probes to detect oxygen consumption rates:* In order to assess the immediate effect of reduced levels of glucose on oxygen consumption rates, the MitoXpress Xtra system from Luxcel Biosciences was used. The assays were carried out according to the manufacturer's protocol using Black 96 well plates with clear bottoms (BD Falcon) and the assays read using a FLUOstar Omega plate reader (BMG LABTECH Ltd). Integration start times of 30µs and 70µs were used with a measurement integration time of 30µs. The data was processed using the script mode function on the BMG FLUOstar Omega reader. Oxygen consumption rates were measured in suspension in media containing 0mM, 1mM, 2.5mM, 5mM, 10mM or 15mM glucose: for INS-1 and HepG2 cell lines, cells were grown in standard cell culture media, then washed with PBS and 100,000 cells were incubated per well in 150µl of media containing different concentrations of glucose. Using the MitoXpress Xtra assay, the

maximal rate of oxygen consumption was determined by calculating the relative rate of oxygen consumed during the linear phase of the assay. Results are expressed as the change in relative fluorescence units (RFU)/min/100,000 cells. [Supplementary Figure 2](#) gives an example of the oxygen consumption rate calculation. Previous studies have directly compared the rates of mitochondrial respiration, obtained with the MitoXpress probe and a Clark electrode approach. When assays are carried out under the same oxygen concentrations (20% oxygen) comparable rates for the removal of oxygen were obtained<sup>35</sup>. The mitochondrial uncoupler FCCP was used to give an indication of the maximal possible rates of respiration.

Although, the Seahorse XF Analyzer (Agilent) system has a higher publication profile, the MitoXpress Luxcel probe systems to measure O<sub>2</sub> consumption and extracellular acidification are becoming increasingly adopted by many researchers<sup>36-39</sup>. This is because it has significant advantages over the Seahorse system including compatibility with many plate readers, multiplexing and when using the MitoXpress intra probe, experiments can be run over several days.

Here we also carried out assays on attached cells using the Luxcel MitoXpress Intra probe, to further compare the oxygen utilization profile of INS-1 cells to other tumour cell lines under different oxygen concentrations. Using this probe (see [Supplementary figures: Figure 3](#) for a typical intracellular O<sub>2</sub> experiment) intracellular O<sub>2</sub> levels can be assessed in cells in real time over prolonged periods (i.e. hours or days). Using a plate reader such as the BMG Omega or Clariostar drugs and environmental conditions such as O<sub>2</sub> can be varied throughout the experiment and the impact on mitochondrial function determined. Cancer cell lines commonly show the Warburg effect, metabolizing glucose via aerobic glycolysis under standard culture conditions (i.e. not using OXPHOS). Cells were plated (50,000 per well) and left to adhere for 6 hours before Luxcel MitoXpress Intra probe was added and cells incubated overnight (5% CO<sub>2</sub>, 20% O<sub>2</sub>). Cells were washed in PBS and media changed as follows: INS-1, 11mM glucose; HepG2, 25mM glucose; and U87MG, 25mM glucose. All treatments were in triplicate wells. Antimycin A (1µM) was used to inhibit oxygen consumption. Using the plate reader's atmospheric control unit and with the plate lid off, the oxygen levels was reduced in a step wise manner. The conversion of fluorescent values to lifetime fluorescence values<sup>40</sup> reduces assay noise and allows the construction of an oxygen calibration curve with a particular lifetime value reflecting a specific oxygen concentration. The antimycin A treated wells, where mitochondrial respiration was inhibited, were used to construct a calibration curve so that intracellular oxygen concentrations could be quantified from lifetime fluorescence values at each oxygen concentration. Percentage intracellular oxygen concentrations were calculated from the following equation as described previously<sup>41</sup> [% O<sub>2</sub>] = A exp(-BT) where the numerical values of the parameters A = 370, B = 0.1 µs<sup>-1</sup>, and T = lifetime value (µs).

**Mitochondrial respiratory control ratio (RCR) measurements**  
Mitochondria from MIN-6 and INS-1 cells were isolated as follows. Cells were grown to 70% confluency in 3 × 175cm<sup>2</sup> tissue culture flasks and washed with PBS prior to removal,



using a cell scraper and 12 ml of ice cold isolation buffer (ISO buffer) (0.25M sucrose, 20mM Hepes, 2mM EGTA, 10mM KCl and 1.5mM MgCl). To reduce cellular superoxide levels 100 $\mu$ M Tiron was incubated with cells in culture. Cells were lysed by shearing on ice, using a Teflon glass homogenizer driven by a motorized stirrer (Heidolph). Large debris, including un-fragmented cells, was removed by centrifugation at 1,100 $\times$ g for 10 minutes at 4°C. The mitochondria were pelleted by centrifugation at 11,000 $\times$ g for 10 minutes at 4°C, washed once with 5 ml of ISO buffer and centrifuged at 11,000 $\times$ g for 5 minutes. Mitochondrial pellets were re-suspended in 300–500 $\mu$ l of ice cold oxygen consumption buffer (0.25M sucrose, 5mM MOPS, 5mM KH<sub>2</sub>PO<sub>4</sub> and 5mM MgCl<sub>2</sub>). Mitochondrial protein levels were determined using a BCA Protein Determination kit (Pierce) and mitochondria introduced into the oxygen consumption chamber (Hansatech) at a final concentration of 0.5 mg/ml. De-fatted BSA (Sigma) was added to the oxygen consumption buffer to give a final concentration of 0.1%. State 4 (Substrate alone: Glycerol-3-Phosphate (G-3-P) 5mM) and state 3 (substrate + 1mM ADP) respiration rates<sup>42</sup> were determined for each mitochondrial prep, and the respiratory control rates (RCR) calculated. G-3-P was used as a substrate, due to its high rate of mitochondria oxygen consumption in isolated INS-1 mitochondria with previous studies indicating that Glycerol-3-phosphate dehydrogenase is expressed at high levels in pancreatic  $\beta$  cells<sup>43</sup>. G-3-P is taken up by mitochondria using the G-3-P shuttle<sup>44</sup>.

### Cellular ATP assay

*Short term low glucose exposure:* INS-1 cells were plated at a density of 50,000 cells per well and were incubated overnight in normal culture media. The media was removed and cells washed twice in glucose free media. INS-1 cells were subjected to either one hour pre-incubation in media containing 1mM glucose or incubated directly in media containing 0mM, 1mM, 2.5mM, 5mM, 10mM and 20mM glucose. Cells pre-incubated in 1mM glucose were then incubated for a further one hour in the different glucose concentrations. All incubations were carried out at 37°C under 5% CO<sub>2</sub>. At the end of the incubation, media was removed and the ADP/ATP ratio of the cells measured using EnzyLight™ ADP/ATP Ratio Assay Kit (BioAssay Systems), according to the manufacturer's instructions.

### Immunoblotting

Protein was extracted from INS-1 cells using a cell extraction buffer (50mM Tris-HCl (pH 7.4), 150mM NaCl, 1 mM EDTA, 1% Triton, 0.1% SDS), containing Mini Complement protease inhibitors (Roche) and phosphatase inhibitor cocktails 1 & 2 (Sigma) at 4°C. Samples were denatured in standard SDS/PAGE loading buffer and 10 $\mu$ g of protein run on a NuPage 4–12% Bis Tris gel in MOPS buffer (Invitrogen). Proteins were transferred onto Hybond C-extra nitrocellulose membrane (Amersham Biosciences), according to manufacturer's instructions. Blocking was performed using 5% fat-free milk solution in 1 X TBS (0.05% Tween) for one hour at room temperature. The levels of ER stress associated proteins were analyzed using the following antibodies: alpha subunit of eukaryotic initiation factor 2 (eIF2  $\alpha$ ; 9722) and binding immunoglobulin protein (BiP; C50B12) rabbit polyclonal antibodies (both 1:1000, Cell Signalling Technologies); rabbit SOD2 antibody (ab13534; 1:2000; Abcam);

mouse monoclonal antibody to  $\beta$ -actin (ab8226; 1:10,000; Abcam). Specificity of antibodies has been shown previously (see manufacturer's websites for published articles and validation) with each identifying a single band of the appropriate size on western blots. Blots and primary antibodies were incubated over night at 4°C in TBS-T containing 5% BSA. The secondary antibody HRP-conjugated rabbit anti-mouse or goat anti-rabbit antibody (Dako) was incubated for 2 hours at a 1:5000 dilution at room temperature in 1 X TBS. Chemiluminescence detection was conducted with the ECL Plus Western Blotting Detection System (Amersham Biosciences) and X-ray film, according to the manufacturer's instructions. Quantification of bands using densitometry was carried out using image J (version 1.54).

### Measuring autophagy using Image Stream

Image Stream (IS100; Amnis) is a multispectral flow cytometer combining standard microscopy with flow cytometry. It can acquire up to 100 cells/sec, with simultaneous acquisition of six images of each cell, including brightfield, scatter, and multiple fluorescent images. We used the integrated software INSPIRE (Amnis) to run the Image Stream. Cells were removed from flasks using Accutase, as outlined previously. For each experiment, 500,000 cells were stained with Lyso-Id (Enzo Life Sciences; ENZ-51005-500) and live dead marker (Invitrogen; L34955), according to the manufacturer's instructions. Cells were then fixed and permeabilised (ebioscience Fixation and permeabilisation Kits; 00-8222-49 and 0083333-56, respectively) and stained with antibodies to LC3 at 1:100 dilution (Epitomics; rabbit antibody; MAP1LC3A, EP1983Y). This was subsequently stained using a goat anti-rabbit antibody (IgG H+/L; Invitrogen; F-2765) conjugated to FITC at a dilution of 1:400. All antibodies were titrated for Image Stream<sup>45</sup>. Cells were finally suspended in 50 $\mu$ l of buffer (cold PBS with 1% FCS and 0.05% sodium azide) in 0.6 ml microcentrifuge tubes. At least 10,000 cells/experimental sample and 2000 cells/single color control were acquired per sample. The .rif files generated from INSPIRE were analyzed using IDEAS 4.0.735 software (Amnis). Single color control files were used to create compensated files (.cif), followed by generation of data (.daf) files. The single cells were gated for in focus cells based on brightfield gradient root mean square (GRMS) feature (>300 GRMS). Mean bright detail similarity was measured on double positive (LC3<sup>hi</sup> Lyso<sup>hi</sup>) cells. Effects of glucose concentrations were assessed by regression analysis and paired sample t testing.

### Statistical analysis

Statistical analysis was carried out using SPSS version 25. For the sample in [Figure 8](#), normality was assessed by visual inspection of frequency plots, and groups were compared by ANOVA. For all other comparisons, nonparametric tests were preferred (Mann-Whitney U or Kruskal-Wallis test as appropriate) or carried out by ANOVA using bootstrap modification.

### Results

**Insulinoma cell lines are sensitive to media glucose levels with reduced glucose associated with increased mitochondrial ROS production**

Initial experiments showed that  $\beta$ -cells grow poorly when media glucose levels are below 5mM ([Supplementary Figure 4A](#)). Poor growth of MIN-6 cells in 5mM glucose under 20% O<sub>2</sub> could

be improved by reducing the concentration of tissue culture oxygen to 10%, implicating oxygen stress (Supplementary Figure 4B). Further studies of the origins of the oxidative stress showed the ROS resulting from incubation in low glucose medium to be of mitochondrial origin. MitoSox analysis of ROS/mitochondrial superoxide by flow cytometry showed a significant increase in mitochondrial ROS production when cells were incubated in low glucose for 4 hours for INS-1 ( $p < 0.001$ ;  $n = 4$ ) and but not for MIN-6 ( $n = 3$ ) cells (calculated by ANOVA). A representative experiment for each cell line is shown in Figures 1A and B.

Three further experimental approaches suggest that this ROS increase is potentially localized to mitochondria. Firstly, a tendency (not significant) to increase levels of the mitochondrial superoxide scavenger SOD2, was observed under low glucose conditions following a 90 minute glucose challenge (Figures 1C and D). Secondly, cytoplasmic ROS levels measured by Oxyburst Green (Supplementary Figures 5A and B) and dihydroethidium (Supplementary Figure 4C) did not show an increase in total cellular ROS following short term low glucose exposure. Thirdly, the absence of ER stress following prolonged low glucose exposure further suggests that the oxidative stress is contained within the mitochondria (Supplementary Figure 6A and B).

#### Priming GSIS with a low glucose challenge significantly affects INS-1 insulin secretion

In most GSIS protocols, cells are routinely exposed to low glucose prior to a glucose challenge. Because the increase in mitochondrial ROS production on reducing glucose was unexpected, we further explored the effect of low glucose challenge on GSIS. Using a standard GSIS protocol incorporating a one hour pre-incubation in 1mM glucose both MIN-6 and INS-1 showed a sigmoidal glucose stimulated insulin secretion response (Figure 2A). The GSIS responses of the two lines differ, with the MIN-6 cells having a higher basal level of insulin secretion and more rapidly reaching maximal secretion when glucose levels are raised. Both lines secrete similar maximal levels of insulin in response to glucose. In Figure 2B, we demonstrate that subjecting INS-1 cells to a one hour pre-incubation in 1mM glucose prior to the GSIS assay affected the amount of insulin secreted. The level of insulin secreted at 10mM glucose significantly increased (Figure 2B).

#### Exposure of INS-1 cells to low glucose causes an anomalous reduction in mitochondrial respiration

When INS-1 cells were incubated in media with low glucose concentrations, whole cell oxygen consumption rapidly falls (Figure 3A). This contrasts with other cell lines, where reducing glucose levels forces cells to use mitochondrial OXPHOS substrates, such as glutamine, increasing oxygen consumption<sup>46</sup> (Figure 3B). The fact that INS-1 cells exhibit a high level of mitochondrial respiration, even when cultured on media containing high concentrations of glucose (10–15mM), is unusual for cultured cells<sup>47–50</sup>. INS-1 cells appear to have a high requirement for glucose to maintain viability, as they show significantly reduced growth rates and are unable to switch to other substrates, such as pyruvate and glutamine, when glucose levels are reduced (Supplementary Figure 4). This elevated rate of oxygen consumption of INS-1 cells cultured on high glucose media is shown in Figure 4. Intracellular oxygen levels were calculated using an

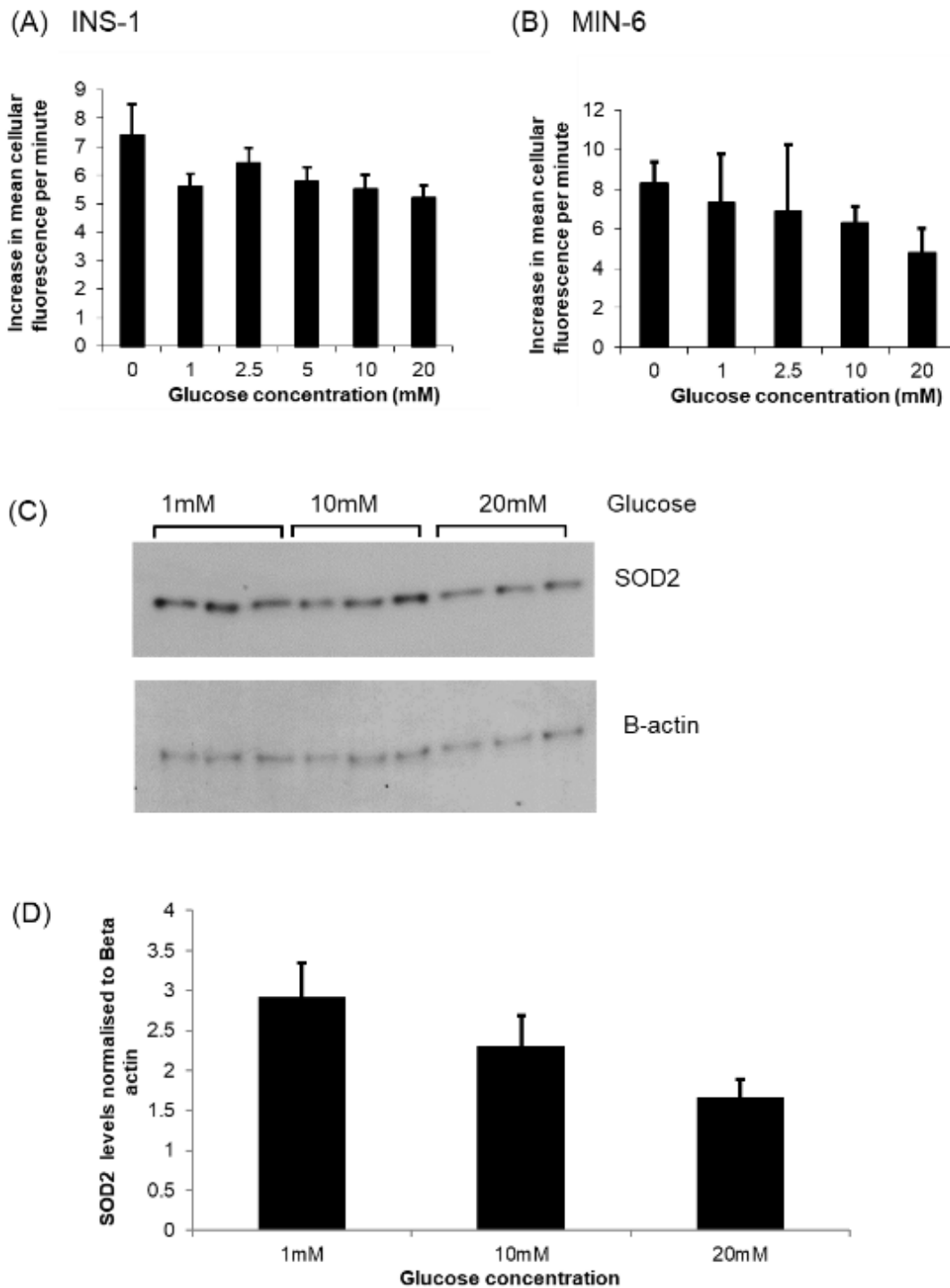
intracellular oxygen probe (MitoXpress, Luxcel Biosciences) and indicate high rates of oxygen consumption occurring in INS-1 cells under a range of atmospheric oxygen concentrations (Figure 4). This contrasts with the more glycolytic U87MG cell line, which consumes very little oxygen under high glucose conditions (Figure 4). Other lines, such as HepG2, also have high respiration rates in high glucose media (Figure 4), but withstand reduced glucose conditions much better, presumably because they can further increase respiration by using other substrates (Figure 3B).

#### ATP levels do not increase in INS-1 cells following a high glucose challenge

In pancreatic  $\beta$ -cells, high glucose exposure increases intracellular levels of ATP which drives the insulin secretion response<sup>17,51,52</sup>. However, in the INS-1 cell line increased levels of insulin secretion and respiration in high glucose media did not correlate with a rise in steady state ATP levels (Figure 5) even though levels of respiration were elevated (Figure 3). ATP levels were actually significantly higher in low (1mM) glucose media compared to 10mM glucose with and without a pre-1 hour low glucose exposure and subsequent glucose challenge (Figure 5). INS-1 cells have very high levels of ATP compared to ADP under all glucose conditions testing making it difficult to observe any meaningful change in the ADP/ATP ratio. (Supplementary Figure 7)

#### Mitochondria in INS-1 cells following prolonged low glucose exposure show reduced metabolic activity, reduced mitochondrial function, whole cell respiration, and down regulated autophagy

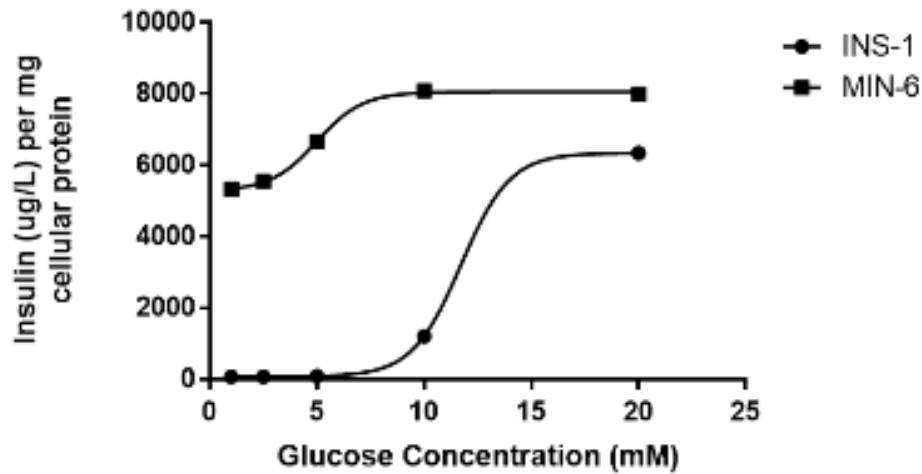
Isolated mitochondria from the INS-1 cells that were subjected to 16 hours incubation in medium containing 1mM glucose showed a significantly reduced mitochondrial respiratory control ratio (RCR; which was decreased to  $2.7 \pm 0.6$  from  $4.9 \pm 0.2$  in 10mM glucose,  $P < 0.01$ ; Figure 6A). This reduction could be rescued by ROS acceptors with the RCR increasing from  $2.7 \pm 0.6$  to  $4.2 \pm 0.4$  in 1mM glucose media on addition of 100  $\mu$ M Tiron (Figure 6A). In 3mM glucose, INS-1 cells required a longer (48 hour) incubation to initiate a significant reduction in mitochondrial RCR levels to  $1.88 \pm 0.7$  ( $P < 0.01$ ; Figure 6A). MIN-6 cells incubated in 1mM glucose showed a similar trend, but this did not reach significance (Supplementary Figure 7). The RCR values for mitochondria prepared from cells growing on low glucose were consistently lower than for cells growing in standard tissue culture glucose concentrations (11mM glucose for INS-1 and 25mM for MIN-6), (Figure 6A and Supplementary Figure 8). These results were supported by measurements of whole cell respiration where cells were incubated for 16 hours in the different substrates (Figure 6B). This differs from experiments in Figure 3A where we determined the immediate effects of switching to different concentrations of glucose on mitochondrial respiration. Whole cell respiration in INS-1 cells is primarily due to mitochondrial respiration because it can be almost completely blocked by the mitochondrial complex II inhibitor antimycin A. This is shown in Figure 4, when treated with antimycin A, intracellular oxygen levels in INS-1 cells reflect atmospheric oxygen. After 16 hours exposure to 1mM and 2.5mM glucose, culture glucose levels were not significantly depleted in INS-1 cells, yet the cells cultured under high glucose depleted the media more rapidly (Table 1). This unexpected result was reflected in the media lactate levels with cells on the high glucose media



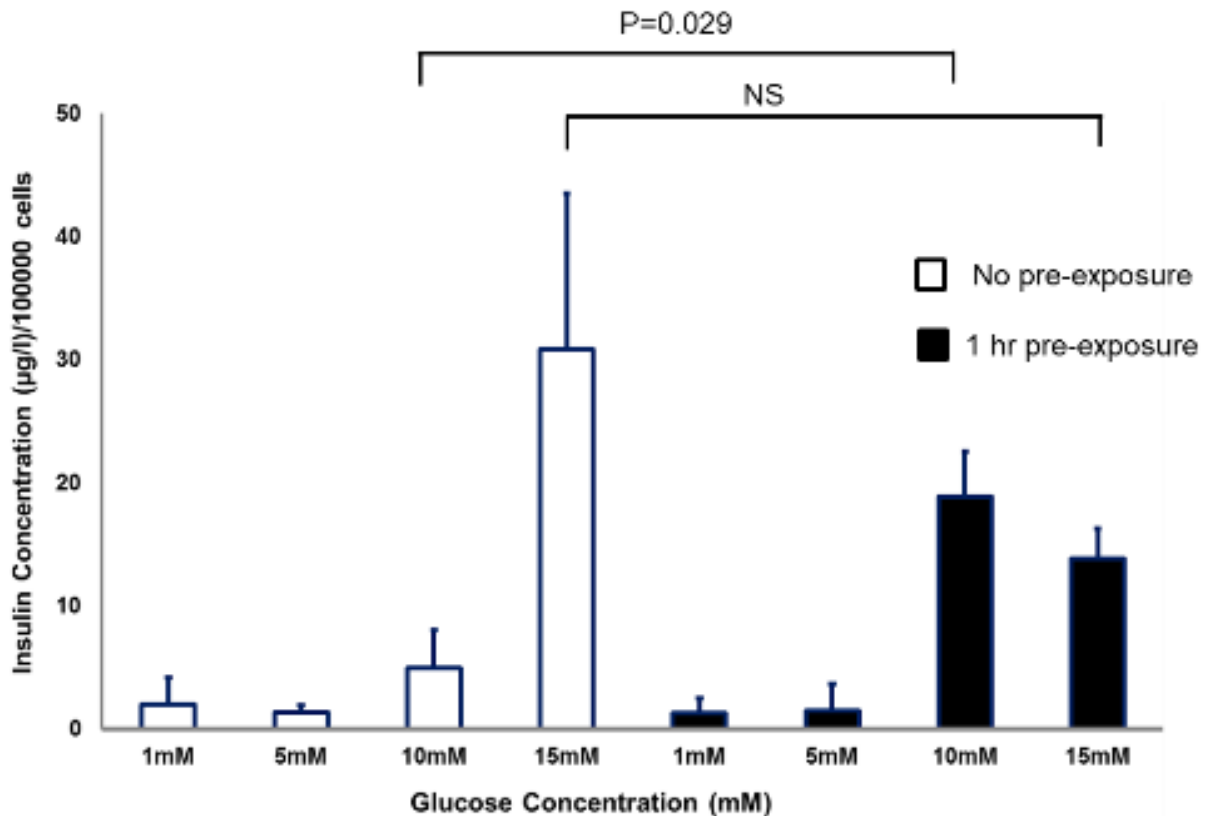
**Figure 1. Low glucose exposure increases beta cell mitochondrial reactive oxygen species (ROS) and antioxidant levels.** MIN-6 and INS-1 mitochondrial ROS production was assessed by loading cells with 5 $\mu$ m MitoSox followed by incubations in different glucose concentrations. **(A)** and **(B)** show the rate of increase in mean cellular MitoSox fluorescence per minute (Ex 488nm, Em 575+ 26nm; 10,000 cells analyzed at each time point) for INS-1 and MIN-6 cells, respectively, for two representative experiments. The log transformed rate of ROS production did not differ significantly from a normal distribution (MIN-6 n=3, INS-1 n=4). This fell significantly with increasing glucose concentration ( $p < 0.01$ ) for INS1 using ANOVA, but was not significant for MIN6. Levels of mitochondrial enzymes involved in controlling mitochondrial superoxide levels were assessed by immunoblotting. **(C)** Immunoblot showing levels of SOD2 levels (molecular weight 26.6KD runs at 24KD on the blot) and  $\beta$ -actin (molecular weight 42KD) following short term (90 minute) reduced glucose exposure of INS-1 cells. **(D)** Levels of SOD2 were determined relative to  $\beta$ -actin using densitometry and Image J software. Experiments were run in triplicate. Statistical comparisons to low glucose conditions (1mM) were made using unpaired t-test. The number of experiments shown in the figure are for biological replicates.



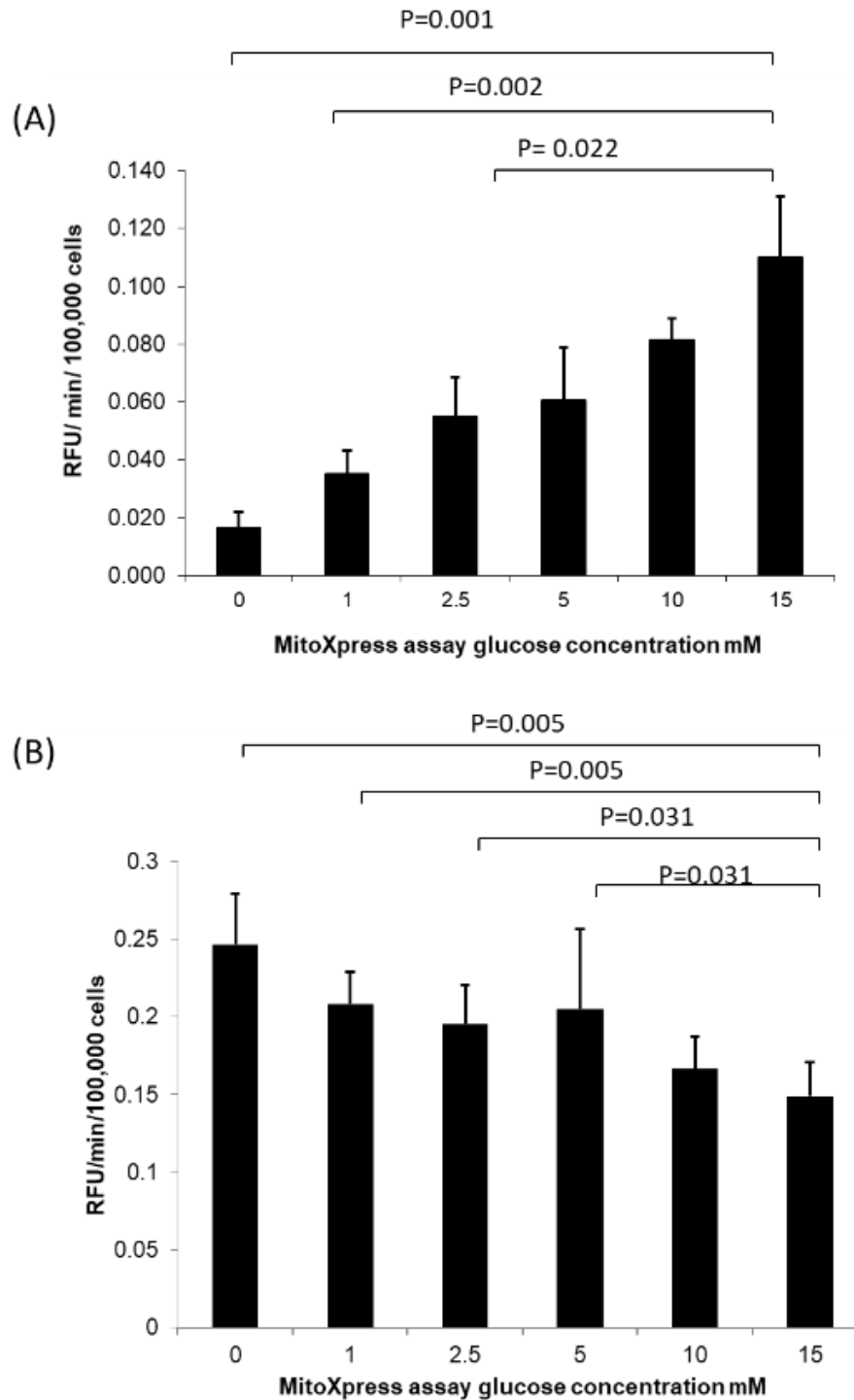
(A)



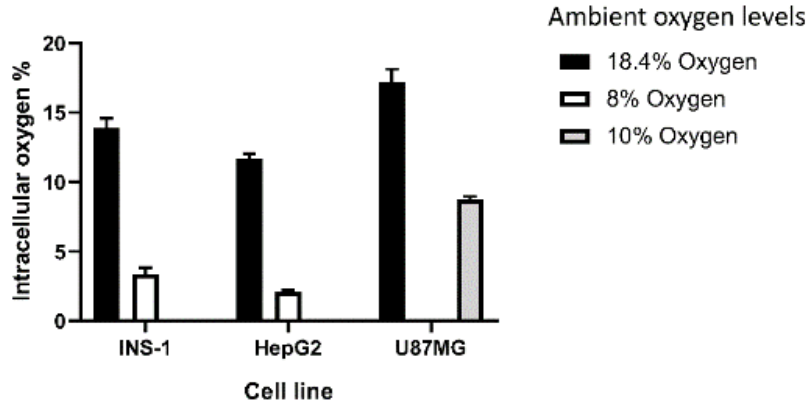
(B)



**Figure 2.** INS-1 and MIN-6 cells show a glucose stimulated insulin secretion (GSIS) response, which is altered by a pre-incubation with low glucose media. (A) MIN-6 and INS-1 cells show a sigmoidal GSIS response. (B) Effect of one hour 1mM glucose pre-incubation on the GSIS response of INS-1 cells (black bars) compared to cells without a pre-incubation (white bars). Data was analyzed using an unpaired t-test. Significant differences were observed when GSIS was compared between the two treatments when cells were incubated in 10mM glucose ( $P=0.029$ ) ( $n=4$  biological replicates).



**Figure 3. Short term reduced glucose exposure over 60 minutes reduces whole cell INS-1 respiration.** Cells were grown under normal culture conditions (11mM glucose for INS-1 cells and 25mM for HepG2 cells), harvested, washed with PBS and incubated in different glucose concentrations containing the MitoXpress Xtra probe to track oxygen consumption. The assay has an initial lag phase of approximately 60 minutes before the maximal rate of oxygen consumption could be determined. **(A)** INS-1 cells show a significant decline in oxygen consumption as glucose levels are reduced. **(B)** This is not observed with HepG2 cells where reduced glucose exposure increases oxygen consumption and is a response to reduced glucose levels well documented in the literature<sup>42</sup>. Significant differences in respiration rates of the different treatments were determined by comparing the rates to those closest to normal culture media glucose levels (10mM INS-1 and 15mM HepG2). One-way ANOVA was used for the analysis; n=18 (3 biological replicates, 6 technical repeats for each day(INS-1)) and n=6 (3 biological replicates, 2 technical repeats for each day (HepG2)).



**Figure 4. INS-1 cells show high levels of oxygen utilization under high glucose conditions.** Intracellular oxygen levels were calculated using the equation  $[\% \text{O}_2] = A \exp(-Bt)$ , where  $A = 370$  and  $B = 0.1 \mu\text{s}^{-1}$ ,  $T =$  lifetime value ( $\mu\text{s}$ ) as outlined in the methods and previously described<sup>41</sup>. Intracellular  $\text{O}_2$  levels for INS-1, HepG2 and U87MG cells were calculated when cells are exposed to ambient 18.4%  $\text{O}_2$ , 8% (INS-1 and HepG2) and 10% U87MG. For the more highly respiring INS-1 and HepG2 cell lines in high glucose media intracellular oxygen levels track below the ambient oxygen concentration in the plate reader as the cells are able to compete against oxygen diffusion into the media. The U87MG cells which have a low rate of respiration in high glucose media are unable to compete against oxygen diffusion into the well and the intracellular oxygen concentration reflects that of the environment (i.e. ambient oxygen concentration). Figure 4 comprises of 6 technical replicates from a single experiment.

producing lactate at a faster rate (Table 1). Accumulation of lactate likely reflects the low activity of lactate dehydrogenase A in INS-1 cells<sup>43,53</sup>. In addition, long term experiments over 16hrs show that although total respiration was impaired under low glucose conditions (Figure 7A), mitochondria under low glucose conditions showed higher rate of maximal mitochondrial respiration (FCCP treatment) relative to the basal respiration rate (Figure 7B;  $p < 0.05$ ) compared to cells on high glucose conditions.

We anticipated that prolonged exposure to reduced glucose would increase autophagy both as a starvation response and for removal of damaged mitochondria. Initial studies using a standard western blot to determine the levels of the activated form of LC3 (which is linked with autophagy) showed INS-1 cells have very high levels of the lipidated LC3 (LC3II) under all glucose conditions (Supplementary Figure 9). Image Stream combines flow cytometry and high-resolution fluorescence microscopy in a single platform, and enables detection of autophagosomes as LC3 punctae co-localizing with lysosomes using bright detail similarity. This method has been validated for detecting autophagy<sup>45,54</sup>. Following incubation we found a significant decrease in the bright detail similarity of co-localizing LC3 and the lysosomal marker, Lyso-ID (autophagy) at low glucose ( $p < 0.02$ ; Figure 8). This was reproducible, bright detail similarity being reduced in cells incubated in 1mM compared to those in 10mM glucose respectively over three runs (paired sample t-test  $P < 0.02$ ). We used HepG2 cells treated with lysosomal inhibitors in starvation medium (HBSS) as a positive control for increased co-localization (Supplementary File 1).

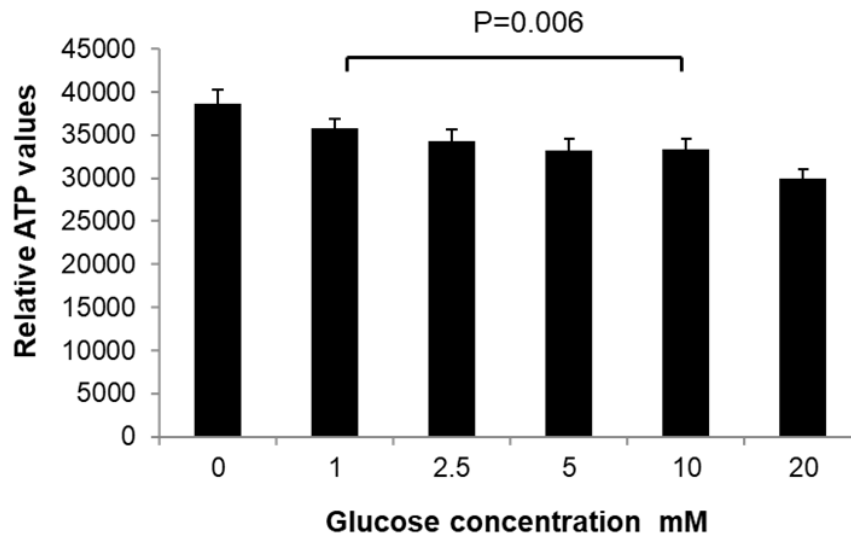
## Discussion

In this study, we induce energetic stress in two insulinoma cell lines, INS-1 and MIN-6 to determine whether they are suitable

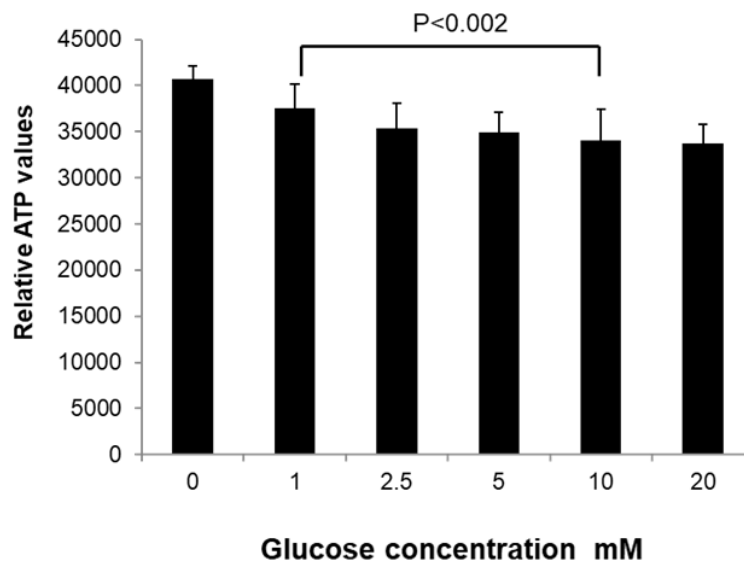
models for studying the effects of mitochondrial dysfunction *in vitro*. Initial studies showed that INS-1 cells have a high rate of mitochondrial oxygen consumption in normal culture media (11–15mM glucose) compared to other cancer derived cell lines, which tend to rely more heavily on glycolysis. High levels of autophagy have recently been associated with high levels of mitochondrial respiration and are shown to be essential for the turnover of damaged mitochondria and maintenance of high ATP levels<sup>55</sup>. Here we show that INS-1 cells have very high levels of autophagy, indicated by high levels of the lipidated autophagy marker LC3-II, further supporting their reliance on OXPHOS or indicating a block in the autophagy pathway. Both insulinoma lines showed a GSIS response and are sensitive to moderate reductions in media glucose ( $< 5\text{mM}$ ); reduced glucose not only resulted in poor growth, but also increased levels of oxidative stress. Unlike previous work showing that oxidative stress caused by high levels of macronutrients (glucose, lipids) and pro-inflammatory cytokines impair  $\beta$ -cell viability<sup>56,57</sup>, the oxidative stress that we documented appears to be confined to the mitochondria. Furthermore, this did not initiate long term ER stress. Within two hours of low glucose exposure, levels of mitochondrial superoxide and superoxide scavenger SOD2 increased. Exposure to high glucose concentrations rapidly elevated mitochondrial respiration, but this did not correlate with an increase in cellular ATP levels, even though an increase in insulin secretion was observed. Prolonged exposure to reduced glucose conditions significantly impaired mitochondrial function and reduced levels of autophagy.

By relying on mitochondrial respiration to trigger the GSIS response, insulinoma lines reflect primary  $\beta$ -cells, which are very sensitive to perturbations of mitochondria function by mtDNA mutations or inhibitors. Previous studies by Sekine *et al.*<sup>43</sup> showed that glycolysis and mitochondrial oxidation are closely

## (A) Pre-incubation with a 1hr 1mM glucose challenge



## (B) No pre-incubation



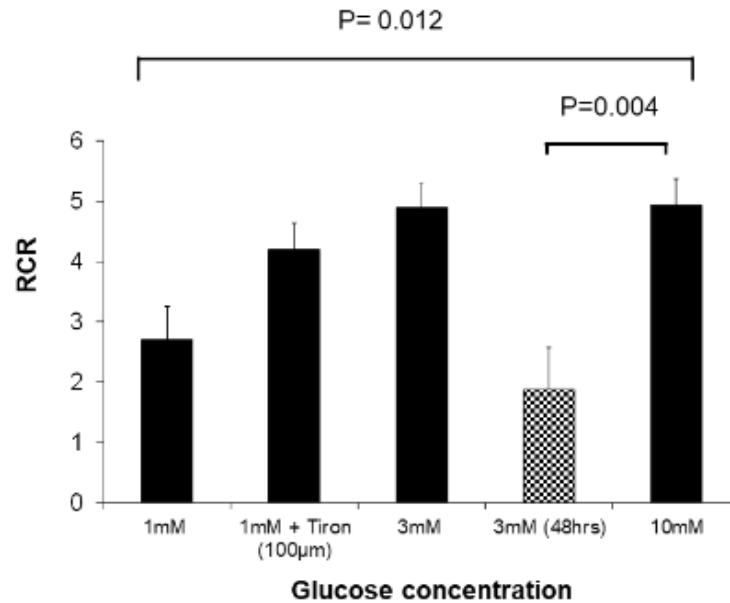
**Figure 5. Steady state ATP levels are increased following a low glucose challenge.** Steady state ATP levels in INS-1 cells following a glucose challenge. **(A)** INS-1 cells are subjected to a 1hr pre-incubation in 1mM glucose prior to glucose exposure. **(B)** INS-1 cells are incubated directly in the final concentrations of glucose without pre-incubation in 1mM glucose. Statistical analysis was carried out using ANOVA on three independent biological experiments. Values were compared to the 10mM glucose samples, as this is closest to normal culture RPMI media glucose levels.

coupled in INS-1 cells. Others have linked the GSIS profile<sup>58</sup> and resulting  $\beta$ -cells viability<sup>59</sup> to glucokinase activity. Our data also indicates that the GSIS profile is affected by glucose conditions immediately before the glucose challenge. A pre-incubation in 1mM glucose increases the levels of insulin generated under moderate glucose conditions (10mM), but reduces insulin secretion under high glucose conditions (15mM). Previous studies

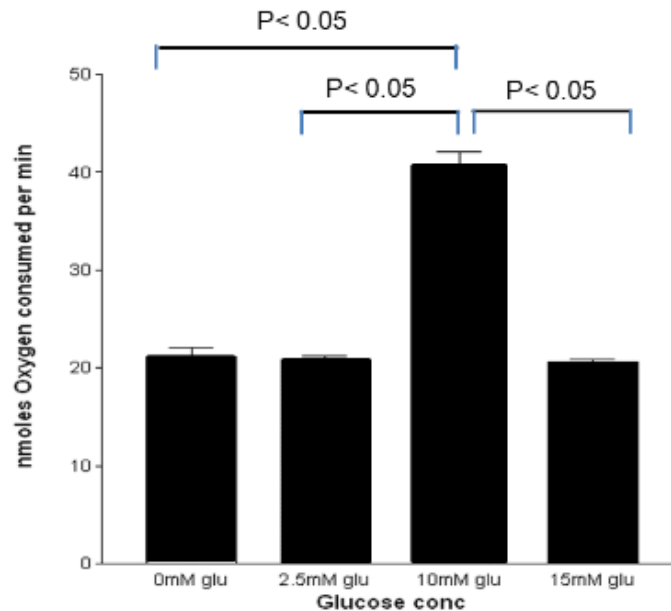
have shown that ROS can trigger insulin secretion, Hence, further studies are required to determine whether the high mitochondrial ROS or the variation in low glucose concentrations is the major priming event for subsequent GSIS. As glucose conditions are constantly fluctuating *in vivo*, it is possible that the  $\beta$ -cell response to low glucose is used to prime the insulin secretion response when glucose levels are raised. While mitochondrial uncoupling



## (A) INS-1 cells



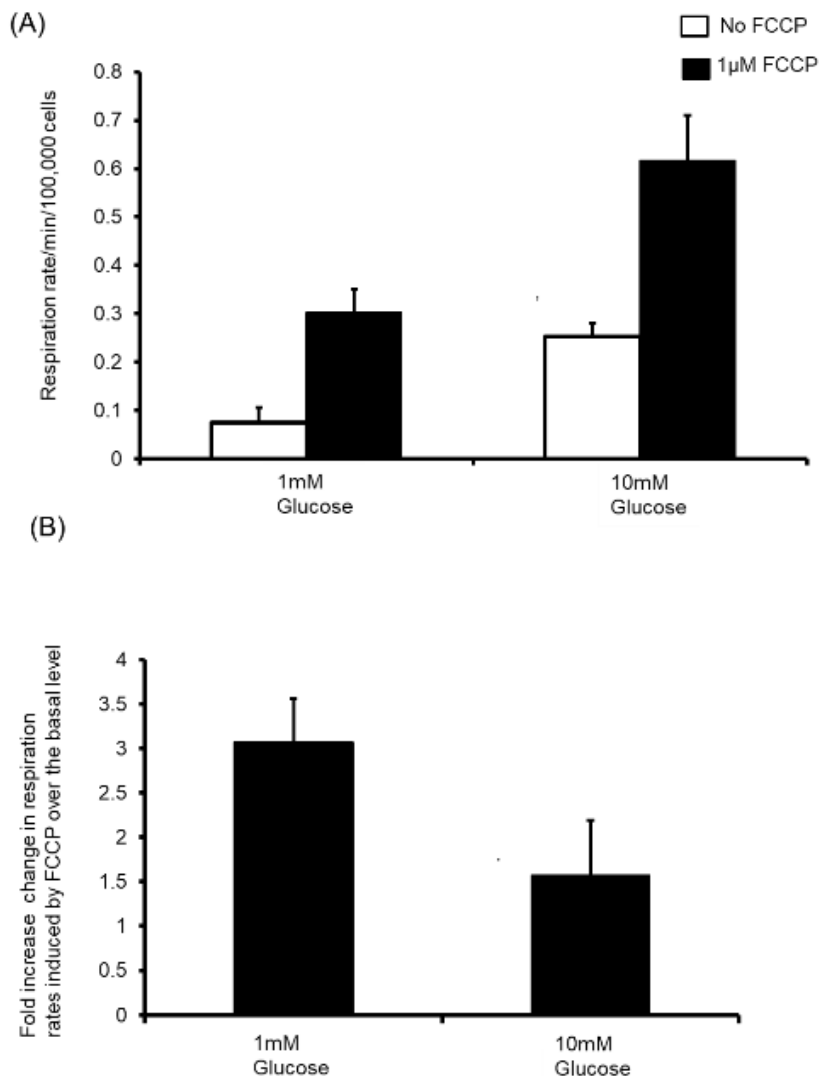
## (B) INS-1 whole cell respiration



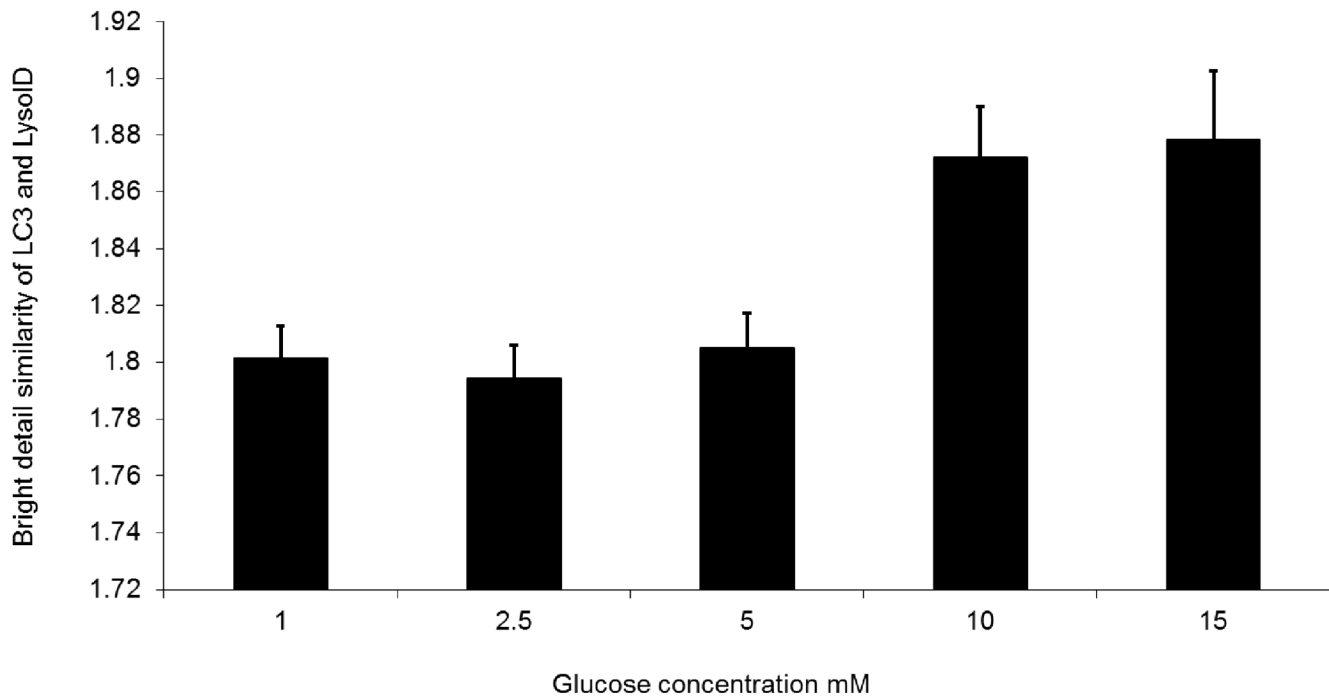
**Figure 6. Effect of low glucose exposure on INS-1 mitochondrial function. (A)** INS-1 cells were incubated for 16 hours in 1mM glucose, 1mM glucose + 100µM Tiron and 3mM and 10mM glucose. 48 hour exposure was also carried out on INS-1 cells incubated in 3mM glucose (checked bar). Isolated mitochondrial respiratory control ratios (RCRs) were compared for each treatment (n=3). **(B)** Whole cell respiration in INS-1 cells (4 million) using an oxygen electrode following 16 hour exposure to different levels of glucose (n=3). Mean cell respiration rates are compared to those obtained under optimal culture glucose conditions (10mM glucose). Data were analysed using a Mann whitney test. Both data sets were from 3 biological replicates.

**Table 1. Media glucose and lactate levels following a 16 hour incubation of INS-1 cells under different glucose concentrations.** (Base line media lactate concentration at time 0=1mM; n=3).

Sample numbers	Mean			Mean		
	Glucose added to media	Media glucose concentration mM 16hrs	STD	Media lactate concentration mM 16hrs	STD	
1,6,11	1mM	1.5	0	1.1	0.21	
2,7,12	2.5mM	2.6	0.15	1.1	0.17	
3,8,13	5mM	4.5	0.41	1.6	0.15	
4,9,14	10mM	8.2	0.64	2.4	0.55	
5,10,15	15 mM	12.3	1.39	2.8	0.71	



**Figure 7. Exposure to reduced glucose conditions increases the spare respiratory capacity.** To determine whether the increased reactive oxygen species (ROS) production and reduced whole cell respiration mitochondrial dysfunction could be linked to changes in the control of OXPHOS, we determined whether mitochondria demonstrated altered spare respiratory capacity. Whole cell respiration rate of INS-1 cells incubated for 16 hours in 1mM and 10mM glucose was determined in the presence of the mitochondrial uncoupler FCCP. A range of FCCP concentrations were used with 1µM giving the maximum increase in whole cell respiration rate for both 1mM and 10mM treated samples (Supplementary File 1). **(A)** A representative experiment with whole cell oxygen consumption rates determined for 1mM and 10mM glucose incubated samples with and without 1µM FCCP included in the assay. **(B)** The results of three independent experiments with an increase in whole cell respiration rates due to FCCP addition relative to the basal rate. The 1mM glucose samples showed a significantly higher spare respiratory capacity ( $p < 0.05$ , unpaired paired t-test) than cells incubated in 10mM glucose. Statistical analysis was carried out by ANOVA method with bootstrap modification (interaction of glucose concentration with presence of FCCP).



**Figure 8. INS-1 cells cultured under reduced glucose conditions show reduced levels of autophagy.** INS-1 cells were cultured in different concentrations of glucose (1, 2.5, 5, 10 and 15mM) for 16 hours and stained for anti-LC3 (autophagosome marker) and Lyso-Id (lysosome marker). The cells were run on Image stream, and 100,000 cells were acquired/treatment. Bright detail intensity was measured on double positive (LC3<sup>Hi</sup> Lyso<sup>Hi</sup>) single in focus live cells. Exposure to reduced glucose significantly decreased the bright detail similarity of co-localizing LC3 and the lysosomal marker, Lyso-ID ( $p < 0.02$ ). This was reproducible: bright detail similarity being reduced in cells incubated in 1mM compared to those in 10mM glucose respectively over three runs (unpaired test;  $p = 0.02$ ).

is already known to modulate insulin secretion<sup>24</sup>, the potential role of ROS in priming of the  $\beta$ -cell to respond to a subsequent glucose challenge is a novel finding. The link between an increase in mitochondrial ROS and increased insulin secretion is supported in studies that show that increased mitochondrial ROS production can increase insulin secretion<sup>18,24</sup>.

ROS increases did not result in an ER stress response. Indeed, the converse was true with eIF2-alpha phosphorylation increasing in high glucose media. Cancer cell lines in general show reduced levels of mitochondrial respiration following prolonged incubation in media containing high concentrations of glucose<sup>27,60</sup>. Reducing glucose concentrations  $< 1$ mM drives the cancer cells to use other energy substrates, such as glutamine and fat, to fuel mitochondrial respiration<sup>39,46,61</sup>. In contrast, insulinoma INS-1 cells do not increase OXPHOS in response to low glucose (Figure 3). Furthermore, exposure of INS-1 cells to 15mM glucose results in an immediate stimulation of mitochondrial respiration which is lost by 16 hours (Figure 6 (B)). Given that INS-1 cells are normally grown in RPMI which has a glucose concentration of 11mM, it is likely that prolonged exposure to 15mM glucose is deleterious to INS-1 cells and hence mitochondrial respiration falls. Moreover, *in vitro* experiments using  $\beta$ -cell lines and isolated pancreatic islets are generally carried out under non-physiological oxygen conditions (20%) which could impact on mitochondrial function and ROS production<sup>62,63</sup>. Further studies of

other beta cell models including bHC, BRIN-BD11, Blox5 which show a strong GSIS response are needed to determine whether our findings reflect general properties of beta cells that are required for their physiological role. An intriguing finding with INS-1 cells is that glucose stimulation did not result in an increase in steady state ATP levels or the ATP/ADP ratio, whether or not there was a one hour pre-incubation in 1mM glucose. Similar results were found by Sarre and colleagues<sup>64</sup>, who also showed that ATP levels were not elevated during GSIS assay. As steady state levels of ATP do not appear to rise in INS-1 cells following glucose stimulation, it is possible that additional mitochondrial secretalogues are generated by increases in mitochondria respiration<sup>14</sup> and is sufficient to drive the increases in insulin secretion in INS-1 cells rather than a global increase in cellular ATP. Increases in ATP could be more localized to the sites of insulin release with no significant changes seen at the cellular level as cell anabolism increases to produce more cellular constituents ready to proliferate. Such changes would not be observed using the techniques employed in this study.

ATP/ADP ratios proved difficult to interpret due to the very low levels of ADP detectable in the INS-1 cell line. The fact that ATP levels remain high in  $\beta$  cells exposed to low glucose with little glucose utilization suggests that INS-1 cells have a mechanism that can conserve ATP levels or generate ATP more efficiently. This could be simply linked to reduced cellular energy

demands under low glucose conditions (i.e. minimal insulin secretion), which may account for the observed net rise in cellular ATP. Alternatively, the mitochondria may be better coupled in low glucose. Interestingly, knockout of UCP2 in  $\beta$  cells increases the amount of insulin secreted under high glucose conditions, highlighting the need for controlling mitochondrial energy production even when the  $\beta$  cell is operating under conditions of high energy need<sup>24</sup>.

In conclusion, our data suggests that insulinoma cell lines show a clear requirement for glucose driven mitochondrial OXPHOS linked to insulin secretion. This is unlike most cancer lines, where glucose suppresses mitochondrial OXPHOS and causes the cells to rely on glycolysis for the generation of ATP and cell growth. Inducing energetic stress by reduced glucose exposure resulted in mitochondrial dysfunction and reduced autophagy over 24 hours, which was associated with increased ROS production. In the short term, pre-incubation with low glucose modulated the insulin secretion response, inducing cells to respond to more physiological levels of glucose at 10mM. This data makes insulinoma cell lines very attractive models to investigate perturbations of mitochondrial function by knocking out genes that cause mitochondrial diseases. Compounds, including Zidovudine, that reduce the levels of mitochondrial DNA could also be studied to investigate the mitochondrial mutation threshold effects observed in mitochondrial disease patients *in vivo*. The lack of an increase in ATP or the ATP/ADP ratio upon glucose stimulation is in contrast

with the canonical increase in ATP or ATP/ADP ratio following a glucose challenge to  $\beta$ -cells. As cancer cells, INS-1 cells are likely to have an improved stress survival response compared to primary cells, and this may explain their ability to withstand long term substrate withdrawal and enter a metabolically quiescent state, which maintains levels of cellular ATP, without triggering ER stress or increasing autophagy.

### Data availability

Raw data for all figures, as either excel spread sheets, prism files or uncropped immunoblots, are available at figshare: doi: [10.6084/m9.figshare.5395453](https://doi.org/10.6084/m9.figshare.5395453)

### Acknowledgments

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### Supplementary files

#### Supplementary Figure 1. Optimisation of mitoSox concentrations to monitor mitochondrial ROS production using flow cytometry.

A ten minute incubation with 5 $\mu$ M mitoSox produced a linear increase in mitochondrial ROS when cells were incubated under ROS inducing low glucose conditions. Panels (A) and (B) show the effect of incubating MIN-6 and INS-1 cells with differing concentrations of mitoSox for 10 minutes prior to ROS induction with 0mM glucose. 0mM glucose was used as this gives the max induction of mitochondrial ROS in the INS-1 and MIN-6 cell lines. The mean cellular fluorescent values were the mean of 10,000 cells counted by flow cytometry. Incubation with 1 $\mu$ M or 0.5 $\mu$ M mitoSox for 10 minutes failed to detect the increases in superoxide above background levels.

[Click here to access the data.](#)

**Supplementary Figure 2. Example of how whole cell respiration rates are calculated using MitoXpress xtra.** An example of the raw data used to calculate the relative rates of oxygen consumption (MitoXpress assay) when INS-1 cells are exposed to low glucose is shown above. Rates of oxygen consumption are determined in the linear portion of the assay (blue box) when the rate of oxygen consumption is at its maximum.

[Click here to access the data.](#)

**Supplementary Figure 3. A typical intracellular O<sub>2</sub> experiment using HT1080 cells and MitoXpress Intra.** A typical intracellular O<sub>2</sub> experiment using HT1080 cells and MitoXpress Intra. The probe was loaded into the cells in a 96 well black plate overnight, cells washed and antimycin A added to some of the wells to inhibit mitochondrial respiration. Intracellular O<sub>2</sub> levels are then monitored in real time in a fluorescent plate reader. Fluorescence values are converted to intracellular O<sub>2</sub> levels using a calibration curve and equation. Modulation of O<sub>2</sub> levels in the plate reader allows intracellular O<sub>2</sub> levels to be measured over a range of ambient O<sub>2</sub> concentrations. In HT1080 cells with high levels of mitochondrial respiration intracellular O<sub>2</sub> levels in the cells are consistently lower than the atmospheric O<sub>2</sub> levels and the levels in the antimycin A treated cells.

[Click here to access the data.](#)



**Supplementary Figure 4. The effect of increasing glucose and oxygen concentration on cell growth characteristics.** Growth of MIN6 (squares) and INS-1 cells (triangles) over 3 days in media containing 0–20mM glucose (A). Tissue culture oxygen was 20% and CO<sub>2</sub> 5%. Flasks were inoculated with  $1 \times 10^6$  and  $2 \times 10^6$  of MIN-6 and INS-1 cells, respectively. Each data point is the mean of three separate experiments. Panel (B) shows the effect of reduced tissue culture oxygen concentrations on the growth of MIN-6 cells grown in 5mM glucose, 5% CO<sub>2</sub> over an 8 day period. Three separate flasks were used for each oxygen concentration. Significance was assessed using Student's t-test.

[Click here to access the data.](#)

**Supplementary Figure 5. Whole cell peroxide and superoxide production under reduced glucose conditions.** For panels (A) & (B) MIN-6 and INS-1 cells are shown in black and grey bars, respectively. Cells were harvested, washed in PBS and labelled with DCFDA, SE (whole cell peroxide) and incubated in different glucose concentrations as outlined in Methods. The data shown is for one hour incubation. Each data point is the mean of three independent experiments (10,000 events counted for each) and the data expressed as mean fluorescence intensity per cell. In panel (C), whole INS-1 cell superoxide levels following reduced glucose exposure were assessed by monitoring levels of 2-hydroxyethidium. Cells were labelled with DHE for 20mins in normal culture media (RPMI 11mM glucose), washed and then incubated in different concentrations of glucose in RPMI media for 90mins. Experiments were carried out in triplicate and no significant effects were observed.

[Click here to access the data.](#)

**Supplementary Figure 6. Long term reduced glucose exposure does not induce a stress response in INS-1 cells.** Levels and activation of ER stress proteins were determined by immunoblotting whole cell protein extracts from INS-1 cells grown for 24 hours under different glucose concentrations. (A) Levels of BiP and eIF2- $\alpha$  are shown. A comparison with an antibody specific for the phos Ser51 eIF2- $\alpha$  indicated that the higher molecular weight band (\*) is the same size as the phosphorylated form of the eIF2- $\alpha$  protein (B) Shows the effect of the ER stress inducing compound tunicamycin (2 $\mu$ g/ml) on INS-1 BiP levels following 20 hour exposure in 10mM glucose with and without 50 $\mu$ M mecaptopethanol. (C) shows quantification of the relative levels of the phosphorylated (\*) eIF2- $\alpha$  relative to the un-phosphorylated form in INS-1 cells following 24 hours exposure to 1mM, 3mM and 10mM glucose.

[Click here to access the data.](#)

**Supplementary Figure 7. A typical experiment showing changes in ATP and ADP values following a 1hr incubation of INS-1 cells in different glucose concentrations.** The figure shows the very low ADP values under all conditions tested resulting in difficulties in determining the ADP/ATP ratio. NB: Values calculated for the same well.

[Click here to access the data.](#)

**Supplementary Figure 8. MIN-6 cells also show a trend towards reduced mitochondrial function on low glucose media.** MIN-6 cells were incubated for 24 hours in 1mM glucose, 1mM glucose + 100 $\mu$ M Tiron and 10mM glucose. Isolated mitochondrial respiratory control ratios (RCRs) were compared for each treatment. Each treatment shows the mean of three independent experiments with statistical comparisons made using Student's t-test.

[Click here to access the data.](#)

**Supplementary Figure 9. INS-1 cells express high levels of the lipidated form of LC3 associated with the autophagosome.** This makes it very difficult to quantify changes in levels of the lipidated form (LC3-II) relative to the non-autophagosome form (LC3-I) by immunoblotting.

[Click here to access the data.](#)

**Supplementary File 1. Amino acid starvation increases autophagy using ImageStream in HepG2 cells.**

[Click here to access the data.](#)

**Supplementary File 2. FCCP optimization as a mitochondrial uncoupling agent in INS-1 cells.**

[Click here to access the data.](#)

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Current Peer Review Status:



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## Version 3

Reviewer Report 13 November 2019

<https://doi.org/10.21956/wellcomeopenres.16930.r36567>

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**Katherine E. Chapman**

In vitro Toxicology Group, Institute of Life Science, College of Medicine, Swansea University, Swansea, UK

I have no further comments to make.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Genetic toxicology with an emerging interest in mitochondrial dysfunction/toxicity relating to hyperglycaemic conditions.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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## Version 2

Reviewer Report 21 January 2019

<https://doi.org/10.21956/wellcomeopenres.13719.r34379>

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**Katherine E. Chapman**

In vitro Toxicology Group, Institute of Life Science, College of Medicine, Swansea University, Swansea, UK



The study investigated the bioenergetic profiles of two rodent *in vitro*  $\beta$ -cell models in response to “high” and “low” glucose concentrations. The aim of the study was to assess the cell models’ potential for studying mitochondrial dysfunction. A thorough, multiple-endpoint analysis has been used to characterise the dose-dependent effects of glucose on the cell models used. The application of more physiologically relevant glucose concentrations than are routinely involved in *in vitro* cell culture is valuable and may be of interest to diabetes and cancer research.

There were, however, several points identified that might improve the manuscript. These are outlined below.

1. It is appreciated that equivalent human models are currently not robust, although it would be useful to include a further justification for the particular cell lines used over other rodent insulinoma cell lines.
2. The Methods are generally sufficiently detailed. Please clarify the kits or approaches used for media glucose and lactate levels. Please also clarify the provenance of the cell lines. In the “Cell lines” section of the Methods section, it is mentioned that the cell lines were obtained from a colleague. Were these obtained directly from the other individuals mentioned who originally derived the cell lines?
3. Please could further information be included in the “Statistics” section (Methods). Were data tested for their conformation to a normal distribution to justify the use of a parametric statistical test (i.e., t-test)? If not, were data transformed? Please clarify what the error bars on graphs represent (e.g., standard deviation). Please clarify whether the experiments contributing towards “n” values (e.g., n=3) were performed as independent biological replicates on separate days.
4. Please include the sizes of SOD2 and B-actin targets for Immunoblotting (Methods) for completion. It is recommended that a labelled part of the protein standards lane would be included in the Western blot images (Figure 1).
5. In the Results section, maximum numerical value on the y-axis values should be the same within multiple graphs with the same y-axis parameters within the same figure. I would suggest setting the maximum values on these graphs to the same for both within the figure, enabling direct comparison between figures. On Figures 4 and 8, it may be helpful to indicate on the graph significant changes, as on other graphs, for consistency.
6. In Table 1, please clarify the assay used and capitalise the second M in the cell containing “1 mM” (typographical error).

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

No source data required

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Genetic toxicology with an emerging interest in mitochondrial dysfunction/toxicity relating to hyperglycaemic conditions.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Reviewer Report 27 April 2018

<https://doi.org/10.21956/wellcomeopenres.13719.r26875>

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**Philip Newsholme**

School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute (CHIRI), Curtin University, Perth, WA, Australia

The authors have adequately addressed the concerns of this referee.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 06 November 2017

<https://doi.org/10.21956/wellcomeopenres.13719.r26876>

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**Charles Affourtit** 

School of Biomedical and Healthcare Sciences, Plymouth University, Plymouth, UK

In response to a negative reception by 2 reviewers, Morten and colleagues have now made several changes to their manuscript. Its focus is still confused, however, as the authors claim different things at different places. The original version of the manuscript suggested to me quite strongly that the authors were pushing the value of insulinoma cells for studying beta cell bioenergetics. Given the unchanged title and introduction of the revised manuscript, readers could be forgiven to think that the authors are still doing so. From the rebuttals, however, it appears the authors' true aim is to contrast insulinoma cells with non-beta-cell models. Based on comparative data, they conclude that insulinoma cells are the preferred option for studying mitochondrial activity *in vitro*, because these cells can be grown under conditions that are physiologically more relevant than the conditions needed to force other cell types to make ATP in an oxidative manner. My problem with this conclusion is that the bioenergetics of pancreatic beta cells have been characterised much less well than those of other cell types such as liver, muscle, heart, kidney and brain. From the limited data that have been reported (reviewed critically in<sup>1</sup>), it is indeed becoming evident that the energy metabolism of beta cells is unusual, with regulatory and control structures that are not typically found in other cells. For *in vitro* studies on effects of drugs or genetic mutations on mitochondrial function, I would personally choose for well-established systems even though they may have to be cultured under conditions that do not fully reflect physiology. In this respect, I entirely agree with the authors that cell models are generally grown under an oxygen atmosphere and high nutritional supply that differ vastly from those prevalent *in vivo*. The attempt to mimic physiological conditions more closely is of course commendable, but the conditions applied here remain an approximation at best, since glucose and other nutrient levels faced by beta cells fluctuate continuously *in vivo*, and because physiological intracellular oxygen tensions are hard to predict. Moreover, mammalian cells are generally grown in batch cultures, which makes it impossible to precisely control the nutrient, product and gas composition of the growth medium.

As stated above, the current manuscript could still be read as an attempt to reveal new insight in the way beta cell bioenergetics control glucose-stimulated insulin secretion (GSIS). Particularly, the authors show that the mitochondrial production of reactive oxygen species (ROS) increases after a period of glucose deprivation. The associated sensitivity of insulin release to glucose is attributed to this increased ROS. Indeed, ROS have been shown by others to induce insulin secretion, but it remains unclear to me why the elevated ROS after glucose deprivation is not enough to increase basal insulin secretion, and why glucose is still required to boost release. More generally, the presented data are correlative and, at best, offer indirect support for the authors' claim. A different study design would be necessary to establish the

relative importance of ROS and the ATP/ADP ratio as GSIS signals. As argued in my first review, the ATP levels reported here are confounded by a lack of the corresponding ADP concentrations and by compartmentalisation complications of whole-cell nucleotide measurements. In my opinion, the apparent negative effect of glucose on ATP levels should not be interpreted as violation of the classical GSIS model. Our calculation of cytosolic ATP/ADP ratios from PCr/Cr ratios yields values that are bioenergetically meaningful and indeed respond to glucose and mitochondrial uncouplers in ways that are predicted by the classical GSIS model. We will publish our data in the foreseeable future, but emphasise that the difficulty of measuring the ATP/ADP ratio that is relevant for GSIS has been discussed in detail by David Nicholls<sup>1</sup>.

My request for more detail on the applied respirometry methodology has been met, but the authors' rebuttal is somewhat defensive. Although I indeed believe that *careful* application of Seahorse XF technology, accounting for oxygen diffusion and for medium acidification by molecules besides lactate [ref-2,<sup>3</sup>, offers a deeper understanding of cellular ATP fluxes than respiratory measurements alone, I wish to stress that I certainly do not suggest that this platform is a 'one-stop shop' for solutions to all bioenergetic problems. Indeed, complete understanding of the role of energy metabolism in GSIS cannot be obtained from metabolic fluxes alone and requires insight in relevant bioenergetic intermediates as well (see e.g. Gerencser *et al.*<sup>4</sup>). Notably, our efforts to measure cytosolic ATP/ADP ratios accurately are required to quantify the relative bioenergetic control of ATP supply and ATP demand, a study that is underway. Related, I agree with the authors that measuring the intracellular oxygen tension may well offer insight in bioenergetics that obviously cannot be obtained from XF measurements. However, the data presented here do not offer such insight: a relatively low intracellular oxygen level in oxidative compared to glycolytic cells seems an inevitable consequence of differences in respiratory activity. To me, novelty cannot simply be claimed by asserting that nobody has yet published this expected consequence.

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**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Mitochondrial biology, cellular bioenergetics

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

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### Version 1

Reviewer Report 10 April 2017

<https://doi.org/10.21956/wellcomeopenres.11353.r20653>

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#### Philip Newsholme

School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute (CHIRI), Curtin University, Perth, WA, Australia

The article submitted to Wellcome Open Research by Morten KJ is a limited study investigating the ATP and ADP concentration change responses and reactive oxygen species generation, GSIS, and viability in two rodent beta cell cell lines MIN-6 and INS-1 to various glucose concentrations. The data generated and associated conclusions in the present submission are not surprising, as there is a large volume of previously published studies investigating glucose metabolism and stimulation of insulin secretion in various beta cell lines and rodent islets. The present paper does not contribute sufficient new and novel information to the to the existing literature, in the opinion of this referee.

The various advantages and disadvantages of specific beta-cell lines have been expertly reviewed previously<sup>1,2</sup>. Several lines do have high intracellular insulin content (HIT, INS-1), while others have adequate expression of GLUT2 or glucokinase (MIN6, bHC9, BRIN-BD11, CM, TRM-1). However, few are fully responsive (compared to primary islet cells) to a rise in physiological glucose with regard to insulin secretion and include INS-1, bHC, BRIN-BD11, Blox5. This is possibly because other cell lines express strong hexokinase activity, which may promote a higher sensitivity towards lower concentrations of glucose due to the low Km of the enzyme. Indeed some cell lines demonstrate either an inadequate response to glucose stimulation (MIN6, NIT-1, RINm5F, RINr, CRI-G1, In-111), or no response at all (CM, TRM-1)<sup>2</sup>. Nonetheless, the most extensively studied b-cell lines are RINm5F, bTC, MIN6, HIT, INS-1 and

BRIN-BD11<sup>1,2</sup> and have aided in the understanding of pancreatic b-cell biology and functionality.

Some of the most important advances stem from experiments investigating the impact of beta cell toxic agents including high levels of macronutrients (glucose, lipids), reactive oxygen species (ROS), and pro-inflammatory cytokines acting on b-cell viability<sup>3</sup>. Equally important, are the experiments demonstrating the protective effect of specific nutrients including polyunsaturated fatty acids, amino acids and anti-oxidants<sup>3</sup>. The cytoprotective action of various nutrients or other agents appear to be through modulation of the redox status of the cell toward a greater anti-oxidant potential<sup>4</sup>. The lack of detail in the manuscript submitted by Morten KJ with respect to previous publications in the area is of concern and their paper is flawed until the previously published work is cited and the potential limitations of working with cell lines is fully described

With respect to experimental design: 'priming' cells with a low-glucose challenge before Insulin release determination is a standard step in protocols for assaying GSIS in insulinoma cells and pancreatic islets. If cells are not deprived from glucose, they will exhibit low secretory sensitivity to glucose probably owing to an elevated basal insulin release.

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**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.



**I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Author Response 11 Sep 2017

**Joanna Poulton**, University of Oxford, John Radcliffe Hospital, Oxford, UK

We thank the reviewer for suggesting that we discuss previous work, we look forward to citing his two reviews (his references 1 and 2) when they become available. However these important additions do not detract from the main point of the paper, that INS-1 cells are very different from non- $\beta$  cell models that are widely used to study mitochondrial function, his comments usefully show that we should re-focus our discussion.

We feel that he has under-estimated the novelty of our findings hence we have expanded the discussion section. Our findings clearly show that INS1 cells could fill a unique niche for studying the toxic effects of drugs on mitochondrial function because they eliminate the need to change media glucose concentrations. To assess the effects of drugs on mitochondrial function or mitochondrial mutations canonical tumour cell lines are routinely grown in very low glucose or even galactose. For most non- $\beta$  cell lines, these conditions (“energetic stress”) force cells to use their mitochondria, being essential to demonstrate the impact mitochondrial dysfunction has on cell physiology. If experiments are carried out in high glucose media the effects of drugs reducing mitochondrial respiration can be missed. Under high glucose conditions many cells generate sufficient ATP via glycolysis to render the mitochondrial energetic impairments insignificant to cellular function. Glucose depletion is useful in the short term but in long term studies glucose depletion imposes a significant cellular stress complicating the situation making it more difficult to determine the mode of action of a drug or mutation impacting mitochondrial function. INS-1 cells on the other hand maintain a high rate of mitochondrial respiration on high glucose (10mM). Hence INS1 cells are potentially an excellent model for studying the effects of mitochondrial drug toxicity because mitochondrial respiration can be constantly maintained without needing to remove glucose.

We have added references to hexokinase as requested

**Competing Interests:** None

Reviewer Report 10 March 2017

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**Charles Affourtit** 

School of Biomedical and Healthcare Sciences, Plymouth University, Plymouth, UK

Morten and colleagues argue that rodent insulinoma cells may provide valuable insight in the energy metabolism of pancreatic beta cells. Having studied the bioenergetics of INS-1E cells for more than a decade, I agree: mitochondrial activity in these model systems generally aligns well with the activity observed in rodent and human pancreatic islets. Indeed, cell studies from my and other labs have revealed aspects of mitochondrial ATP synthesis that appear specific (albeit not unique) to beta cells and are reproducible in islets – these aspects include a bioenergetic glucose sensitivity and relatively low coupling efficiency of oxidative phosphorylation. Notably, links between oxidative ATP synthesis defects and impaired glucose-stimulated insulin secretion (GSIS) seen under pathological conditions, are evident in both insulinoma cells and islets. In a similar spirit, INS-1 and MIN-6 cells are used here ‘(...) to determine whether they are suitable for studying the effects of mitochondrial dysfunction *in vitro*.’ This suitability is indeed becoming increasingly clear, but I do not think the data presented in this paper strengthen the case. No attempt has been made to validate any finding in primary beta cells or islets, so the physiological relevance of reported mitochondrial dysfunction remains unclear. Furthermore, the respiratory data were mostly obtained with insufficiently explained methodology and are internally inconsistent. Effects of cellular glucose deprivation on the linked respiration and insulin secretion lack novelty, whilst a glucose-induced decrease of ATP level is indeed unexpected, but most likely results from experimental limitations. On the whole, I do not feel that this paper offers the insights in beta cell energy metabolism that its title claims and, regretfully, I am unable to approve it. The following concerns are the main reason for my lack of enthusiasm.

1. The authors report functional effects of ‘priming’ cells with a low-glucose challenge before assays. As far as I know, a fuel deprivation of 1-2 h is a fairly standard step in protocols for assaying GSIS in insulinoma cells and pancreatic islets. As is indeed the case here, insulinoma cells are typically cultured at a glucose level (10-25 mM) that provokes insulin release. If cells are not deprived from glucose, they will exhibit low secretory sensitivity to glucose probably owing to an elevated basal insulin release. The GSIS protocol applied here detects the insulin secreted by cells incubated at various glucose levels for 30 min. However, this measurement window is preceded by a 30-min incubation at the same glucose levels – the insulin secreted during this time is discarded. Although legitimate, this unusual experimental design implies that even the cells that had not been deprived from glucose *before* the assay, were indeed fuel-limited for at least 30 min *during* the assay. From Fig. 1A it would appear that such short glucose restriction is indeed sufficient to lower the basal insulin release of INS-1 cells grown at 10 mM glucose, but fails to dampen the basal insulin secretion of MIN-6 cells that are grown at 25 mM glucose.
2. The authors report that a low-glucose priming period (a 60-min lag phase is mentioned in the legend of Fig. 3) sensitises INS-1 respiration to glucose. Again, typical protocols for insulinoma respiratory assays include incubation of cells without glucose, or at a low glucose level, before oxygen uptake measurements. It has been reported widely that mitochondrial oxidative phosphorylation of insulinoma cells as well as islets is sensitive to glucose under these conditions, and bioenergetic glucose sensitivity underpins nutrient-secretion coupling that characterises pancreatic beta cells<sup>1</sup>. Although the fluorescence units make it hard to compare the INS-1 respiratory data reported in Fig. 3 with published values, and although the data have not been corrected for non-mitochondrial oxygen consumption, glucose stimulation of whole-cell INS-1 respiration is consistent with the literature and is indeed predicted by the classical GSIS model.
3. The MitoXpress assay used to measure cellular oxygen consumption is not explained very clearly and the reader is referred to previous literature for validation of the method by oxygen electrode measurements. Although cell history differs, it is concerning in this respect that the electrode data shown in Fig. 6B (no stimulation of basal respiration by 15 mM glucose) are inconsistent with the

fluorescence data in Fig. 3A (large stimulation by 15 mM glucose).

4. Similarly, the measurement of intra-cellular oxygen concentrations in cultured cells (Fig. 4) is not explained clearly, and it is hard to derive the claimed message from the way the data are presented. If I understand correctly, the authors attempt to relate intra- and extra-cellular oxygen concentrations. Such relations would be clearer from line graphs than from tables, which would facilitate comparison of different cells types. In any case, however, the author's conclusion that intra-cellular oxygen concentration deviates more from the applied ambient oxygen tension in oxidative (e.g. INS-1) than glycolytic cells seems unsurprising.
5. An unexpected finding reported in this paper is the lowering effect of glucose on the total ATP content of 'primed' INS-1 cells. Although the authors rightly assert that it is the ATP/ADP ratio that is bioenergetically relevant, not the ATP level *per se*, such inverse glucose sensitivity would be at odds with the canonical GSIS model in which a glucose-induced rise in the ATP/ADP ratio triggers electrophysiological events that ultimately lead to the exocytosis of insulin-containing granules. Based on our own recent attempts, however, I suspect that this unusual finding may reflect the difficulty of measuring cytosolic ATP/ADP ratios. Indeed, the authors acknowledge explicitly they found it hard to measure accurate ADP levels, which are much lower than the ATP levels. In other words, the little ADP that is converted to ATP during the assay is to be detected against a high ATP background. We have measured ATP/ADP ratios in INS-1E cells using sulphurylase to remove ATP before ADP measurements. With this protocol we obtain total ATP/ADP ratios with values around 5-10, which increase significantly in response to glucose and collapse in the presence of a mitochondrial uncoupler (Carré J.E. and Affourtit C., unpublished). Importantly, however, interpretation of total ATP/ADP values in beta cells is confounded by multiple adenine nucleotide pools of cytosolic, mitochondrial and granular origin, respectively. Cytosolic ATP/ADP ratios can be calculated from phosphocreatine/creatine (PCr/Cr) ratios. From our recent experiments it transpired that INS-1E cells express a creatine kinase as we detected a PCr pool when cells were grown in the presence of 3 mM Cr. Assuming an equilibrium constant for the creatine kinase reaction ( $\text{PCr} + \text{ADP} \rightleftharpoons \text{Cr} + \text{ATP}$ ) of 110, we calculated a cytosolic ATP/ADP ratio of around 70, a value that increased to just below 300 in response to glucose and collapsed in the presence of uncoupler (Wynne A. and Affourtit, C., unpublished).

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**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

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**If applicable, is the statistical analysis and its interpretation appropriate?**

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**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Author Response 11 Sep 2017

**Joanna Poulton**, University of Oxford, John Radcliffe Hospital, Oxford, UK

We thank Dr Affourtit for supporting our conclusion that oxidative ATP synthesis defects is linked to impaired GSIS in INS-1 cells and beta cells, "...a cell type whose unique properties are important in pathogenesis" (opening sentence). However, we did not carry out this study as a way into studying islets, interesting though this direction would be. Instead we wish to contrast INS-1 cells with the non-beta cell models that are widely used to study mitochondrial function, hence our expanded discussion section.

Referees comments shown in italics

*The authors report functional effects of 'priming' cells with a low-glucose challenge before assays. As far as I know, a fuel deprivation of 1-2 h is a fairly standard step in protocols for assaying GSIS in insulinoma cells and pancreatic islets. As is indeed the case here, insulinoma cells are typically cultured at a glucose level (10-25 mM) that provokes insulin release. If cells are not deprived from glucose, they will exhibit low secretory sensitivity to glucose probably owing to an elevated basal insulin release. The GSIS protocol applied here detects the insulin secreted by cells incubated at various glucose levels for 30 min. However, this measurement window is preceded by a 30-min incubation at the same glucose levels – the insulin secreted during this time is discarded. Although legitimate, this unusual experimental design implies that even the cells that had not been deprived from glucose before the assay, were indeed fuel-limited for at least 30 min during the assay*

We designed our experiments to explore the impact of different times of glucose deprivation because of our initial observations that low glucose exposure increased mitochondrial ROS. We wondered if differences in levels of mitochondrial ROS linked to glucose deprivation could impact on the amount of insulin secreted in a subsequent high glucose challenge (Figure 1). The longer period of glucose deprivation increased the amount of insulin secreted at moderate glucose levels (10mM) with a decline at (15mM) compared to cells not subjected to a glucose deprivation challenge (Figure 2). As the increase in mitochondrial respiration driven by increased glucose exposure is very rapid even without a low glucose challenge (Figure 3) we suggests that the increase ROS production due to low glucose exposure could have a role in the type of GSIS response which may not be solely due to increased mitochondrial respiration and increased ATP generation. Interestingly a prolonged incubation (16hrs) in high glucose conditions 15mM eventually inhibited INS-1 oxygen consumption.

We have now changed the order of the manuscript in order to better explain the rational of our

experimental approach. Drugs that both inhibit the mitochondrial respiratory chain and generate ROS, such as rotenone can trigger insulin secretion without increasing the glucose concentration (Leloup et al (2009). Hence the increased insulin secretion could be mediated by ROS and not just ATP. Our studies suggest that exploring the impact of mitochondrial ROS/mitochondrial signalling in the context of insulin secretion is an interesting avenue to explore and may differ from the established link to elevations of ATP driving the insulin secretion response. As the reviewer later points out the lack of a significant rise in ATP with glucose is surprising and may point to other mechanisms playing a role in the GSIS response in INS-1 cells

*From Fig. 1A it would appear that such short glucose restriction is indeed sufficient to lower the basal insulin release of INS-1 cells grown at 10 mM glucose, but fails to dampen the basal insulin secretion of MIN-6 cells that are grown at 25 mM glucose.*

It could be speculated that the different effects observed at 10mM and 25mM glucose are due to the different effects of a short glucose restriction on the two cell lines. Min-6 cells grew less well than INS-1 cells under standard culture conditions (i.e. high glucose and 20% Oxygen). We noticed very early on that MIN-6 cell growth could be improved by culturing cells under reduced Oxygen (supplementary figure 4 B). If ROS levels are important in insulin secretion then perhaps MIN-6 cells are already primed to respond to lower concentrations of glucose. In addition to high glucose (11-25mM) used to study biology in many different types of in vitro systems Oxygen at 20% is significantly higher than that found in vivo. For example O<sub>2</sub> levels around a beta cell in vivo will be likely less than 5% and could therefore have a significant impact on ROS levels and cellular metabolism. Although both referees feel this study does not add much to the current consensus as to how a beta cells function in vitro a key point to consider is how accurately the data generated from cellular in vitro β cell models and isolated pancreatic islets under non-physiological conditions accurately reflect the situation in vivo. In our opinion studies exploring the impact of substrate availability (e.g. glucose and oxygen) is of fundamental importance as we develop new models which more accurately reflect the situation in vivo. This is not only the case for the pancreatic beta cell but also neurons, stem cells and cancer cells all currently cultured in 25mM glucose with 20% oxygen.

*The authors report that a low-glucose priming period (a 60-min lag phase is mentioned in the legend of Fig. 3) sensitises INS-1 respiration to glucose. Again, typical protocols for insulinoma respiratory assays include incubation of cells without glucose, or at a low glucose level, before oxygen uptake measurements. It has been reported widely that mitochondrial oxidative phosphorylation of insulinoma cells as well as islets is sensitive to glucose under these conditions, and bioenergetic glucose sensitivity underpins nutrient-secretion coupling that characterises pancreatic beta cells<sup>1</sup>. Although the fluorescence units make it hard to compare the INS-1 respiratory data reported in Fig. 3 with published values, and although the data have not been corrected for non-mitochondrial oxygen consumption, glucose stimulation of whole-cell INS-1 respiration is consistent with the literature and is indeed predicted by the classical GSIS model.*

It is true that other systems such as the Sea Horse bioanalyser convert the values from a fluorescence detection system into nmoles of O<sub>2</sub> allowing rates of oxygen consumption to be given units with rates calculated over time. A similar detection system is used with the Luxcel MitoXpress probes with the signal quenched by the presence of Oxygen. In Figure 3 we use fluorescence values as an indicator of Oxygen levels in the oil sealed well and from this data calculate a rate of change of fluorescence which reflects the rate of oxygen consumption. In figure 3 we are primarily comparing the difference in oxygen consumption of the two lines rather than making a comparison

with other publications on INS-1 cells. Therefore in the context of this paper we feel our conclusions are valid. The reason rates of oxygen consumption in nmoles of  $O_2$  are not calculated with MitoXpress extra is that we are concerned about differences in back diffusion as oxygen is removed from the sealed well. Oxygen can diffuse through the plastic plate and through the oil. If this occurs at different rates when  $O_2$  levels are at 15% and 5% respectively this will affect the respiration rate which should be adjusted to account for the rate of back diffusion. As we do not know the levels of back diffusion then we cannot accurately give a rate of  $O_2$  consumption per unit time. A similar issue is observed with the Seahorse system with back diffusion around the sealing probe as the well is sealed with a plunger to make the  $O_2$  measurements. As the seal may vary within wells in a plate and between plates the oxygen concentration values are unlikely to 100% accurate. The fast rate of oxygen consumption of the INS-1 cell line in high glucose media is confirmed in figure 4 using the intracellular  $O_2$  probe. Here fluorescence life time rather than fluorescence values are used with an  $O_2$  calibration curve to accurately measure intracellular  $O_2$  levels. Intracellular  $O_2$  levels are much lower than the ambient  $O_2$  levels even when ambient  $O_2$  levels are low further suggesting a high rate of mitochondrial respiration. As shown in figure 4 Antimycin A intracellular  $O_2$  levels for INS-1 closely reflect the ambient oxygen levels of the plate reader supporting the idea that mitochondrial respiration in this line is the primary source of cellular oxygen consumption.

*The MitoXpress assay used to measure cellular oxygen consumption is not explained very clearly and the reader is referred to previous literature for validation of the method by oxygen electrode measurements.*

While less widely used and marketed than Seahorse, we assert that MitoXpress is well validated. We concede that it is less well known and have therefore added the explanation requested as an extra supplementary figure (see supplementary figure 3).

*Although cell history differs, it is concerning in this respect that the electrode data shown in Fig. 6B (no stimulation of basal respiration by 15 mM glucose) are inconsistent with the fluorescence data in Fig. 3A (large stimulation by 15 mM glucose).*

The cells used in Fig 6 would have been grown for a prolonged period > 16hrs in 15mM glucose before  $O_2$  rates were determined. Those in Fig 3 A were measured straight away with the  $O_2$  rate measured within 60 minutes of glucose addition. We have now made this clear in the text. A discussion point has also been added which comments on the potential differences of short and long term 15mM glucose exposure on INS-1 cells in the context of other cancer cell lines. Not only does high glucose exposure reduce mitochondrial respiration in other cancer cell lines following long term exposure it is a concentration 5mM higher than the RPMI media used to grow INS-1 cells. There is the potential for high glucose exposure to impact on INS-1 function which could reduce levels of mitochondrial respiration

*Similarly, the measurement of intra-cellular oxygen concentrations in cultured cells (Fig. 4) is not explained clearly, and it is hard to derive the claimed message from the way the data are presented.*

The calibration curve and subsequent equation are shown in Supplementary figure 2 and this is referred to more clearly in the text with further explanation of how the probe works in a new supplementary figure. Supplementary figure 3 shows the type of intracellular oxygen data



generated at a single O<sub>2</sub> concentration.

*If I understand correctly, the authors attempt to relate intra- and extra-cellular oxygen concentrations. Such relations would be clearer from line graphs than from tables, which would facilitate comparison of different cells types. In any case, however, the author's conclusion that intra-cellular oxygen concentration deviates more from the applied ambient oxygen tension in oxidative (e.g. INS-1) than glycolytic cells seems unsurprising.*

Although we have taken key O<sub>2</sub> values at specific oxygen concentrations from the curves to generate the tables. The curves shown giving intracellular oxygen levels in the presence of antimycin A show the actual data and are indicated on the graphs on Figure 4. Maybe unsurprising to this reviewer but novel given that no one has previously published on this. Furthermore the switching between oxidative and glycolytic metabolism might affect intracellular O<sub>2</sub> conc and hence signals such as HIF activation is widely overlooked. We have therefore expanded this in the discussion section. We assert that the Mitoxpress intra probe will be an important tool in future research into cell metabolism and bioenergetics. The probe can be used in real time assays over hrs or days and be multiplexed with other assays include JC1 (Caymen) and cell tox green (Promega) in plate based assays. In this it offers significant advances over the Sea Horse system. Figure 4 in the manuscript was obtained by loading cells with Mitoxpress intra and then measuring intracellular oxygen levels with cells grown in high glucose media at different O<sub>2</sub> levels. The glycolytic line U87MG behaves very differently in low glucose media where cells switch to mitochondrial respiration Potter et al (2016).

*An unexpected finding reported in this paper is the lowering effect of glucose on the total ATP content of 'primed' INS-1 cells. Although the authors rightly assert that it is the ATP/ADP ratio that is bioenergetically relevant, not the ATP level per se, such inverse glucose sensitivity would be at odds with the canonical GSIS model in which a glucose-induced rise in the ATP/ADP ratio triggers electrophysiological events that ultimately lead to the exocytosis of insulin-containing granules.*

We are pleased that the reviewer finds this key finding intriguing. The cells in the reduced glucose concentration also show reduced lactate production which could be indicative of a reduce glycolysis with the cells moving into a quiescent state. Under the low glucose conditions where cells appear to be carrying out less glycolysis (i.e. reduced lactate) other substrates could be utilised by mitochondria as a fuel. As growth rates drop markedly in INS-1 cells when cultured in reduced glucose media see supplemental figure 3 the reduced growth and ATP generated from non-glucose substrates could result in a net increase in total ATP. The net increase in ATP observed with short low glucose exposure compared to high (20mM) (1hr) shown in figure 5 is also observed after 24hrs (data not shown).

*Based on our own recent attempts, however, I suspect that this unusual finding may reflect the difficulty of measuring cytosolic ATP/ADP ratios.*

*Indeed, the authors acknowledge explicitly they found it hard to measure accurate ADP levels, which are much lower than the ATP levels. In other words, the little ADP that is converted to ATP during the assay is to be detected against a high ATP background. We have measured ATP/ADP ratios in INS-1E cells using sulphurylase to remove ATP before ADP measurements. With this protocol we obtain total ATP/ADP ratios with values around 5-10, which increase significantly in response to glucose and collapse in the presence of a mitochondrial uncoupler (Carré J.E. and Affourtit C., unpublished). Importantly, however, interpretation of total ATP/ADP values in beta cells is confounded by multiple adenine nucleotide pools of cytosolic, mitochondrial and granular origin,*

respectively. Cytosolic ATP/ADP ratios can be calculated from phosphocreatine/creatine (PCr/Cr) ratios. From our recent experiments it transpired that INS-1E cells express a creatine kinase as we detected a PCr pool when cells were grown in the presence of 3 mM Cr. Assuming an equilibrium constant for the creatine kinase reaction ( $PCr + ADP \rightleftharpoons Cr + ATP$ ) of 110, we calculated a cytosolic ATP/ADP ratio of around 70, a value that increased to just below 300 in response to glucose and collapsed in the presence of uncoupler (Wynne A. and Affourtit, C., unpublished).

This is an important point as we have had problems using ATP/ADP kits using other models (i.e. primary rat foetal neurons). If the Affouritt data is published we would be glad to reference it in the paper as the problems using these ATP/ADP ratio assays needs to be pointed out and alternative approaches highlighted. Changes in AMP which are larger than those for ADP and ATP maybe a better indicate of metabolic switching as cells transition from one carbon source to another.

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**Competing Interests:** None