Video Article

High-resolution Respirometry to Measure Mitochondrial Function of Intact Beta Cells in the Presence of Natural Compounds

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Abstract

High-resolution respirometry allows for the measurement of oxygen consumption of isolated mitochondria, cells and tissues. Beta cells play a critical role in the body by controlling blood glucose levels through insulin secretion in response to elevated glucose concentrations. Insulin secretion is controlled by glucose metabolism and mitochondrial respiration. Therefore, measuring intact beta cell respiration is essential to be able to improve beta cell function as a treatment for diabetes. Using intact 832/13 INS-1 derived beta cells we can measure the effect of increasing glucose concentration on cellular respiration. This protocol allows us to measure beta cell respiration in the presence or absence of various compounds, allowing one to determine the effect of given compounds on intact cell respiration. Here we demonstrate the effect of two naturally occurring compounds, monomeric epicatechin and curcumin, on beta cell respiration under the presence of low (2.5 mM) or high glucose (16.7 mM) conditions. This technique can be used to determine the effect of various compounds on intact beta cell respiration in the presence of differing glucose concentrations.

Video Link

The video component of this article can be found at https://www.jove.com/video/57053/

Introduction

The primary purpose of the pancreatic beta cell is to maintain system normoglycemia through glucose-stimulated insulin secretion. The beta cells sense physiological changes in circulating glucose largely due to the low affinity, high capacity glucose transporter GLUT2 (Glucose Transporter 2, K_m 16.7 mM)[1]. As circulatory glucose levels rise, this high-capacity low-affinity transporter facilitates a proportional increase in intracellular glucose within the beta cell. Glucose is metabolized through glycolysis, the TCA cycle (tricarboxylic acid cycle) and mitochondrial respiration resulting in elevated cellular ATP (adenosine triphosphate) levels. The elevated ATP concentration blocks the ATP sensitive K^+ channels, resulting in membrane depolarization. Membrane depolarization causes the opening of voltage gated Ca^{2+} channels and subsequent release of vesicle bound insulin granules[2]. Beta cell dysfunction is a hallmark of Type 2 Diabetes (T2D), and results in decreased and poorly controlled insulin secretion and ultimately beta cell death[3]. Mechanisms that maintain or improve beta cell function could be used as a treatment for T2D.

Studies have demonstrated the beneficial effects of naturally occurring plant-based compounds on the pancreatic beta cell[4]. These compounds may have their effect through increasing beta cell proliferation, survival, or glucose-stimulated insulin secretion. As an example, recent studies have demonstrated that monomeric epicatechin enhances glucose-stimulated insulin secretion through increasing mitochondrial respiration and increasing cellular ATP levels[5]. Therefore, understanding how these compounds can increase functional beta cell mass is important to leverage these compounds as potential therapeutics.

Cellular respiration can be measured through a number of tools. Use of a high-resolution respirometer allows for titration of chemical modulators to a permeabilized or intact cell population[6]. This tool permits the addition of various compounds, at different concentrations, thus giving a wide array of information.

Given the intimate connection between glucose metabolism and beta cell function, measurements of cellular respiration are critical. Measurements of cellular respiration can be done using either permeabilized or intact beta cells, with each having its own set of benefits and drawbacks[7,8]. While permeabilization of beta cells allows one to measure different aspects of the electron transport chain, it does so without regards to the mechanism for inducing respiration in the beta cell, glucose uptake and metabolism. Therefore, use of unpermeabilized beta cell respiration is a very useful technique to determine the beta cells response to various glucose levels, using oxygen consumption as the readout.

The purpose of this technique is to measure oxygen consumption in intact INS-1 derived 832/13 beta cells. This technique allows us to determine the response of the beta cells to unstimulatory glucose conditions (2.5 mM glucose) as well as stimulatory glucose conditions (16.7 mM glucose). While the unpermeabilized cells do not allow us to individually test complex I, II or III of the electron transport chain, the technique does permit measurements dealing with complex IV inhibition (Oligomycin A), uncoupled respiration (FCCP-Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone), and completely inhibited respiration (Antimycin A). This study demonstrates the efficacy of measuring

respiration in intact unpermeabilized pancreatic beta cells, as well as the effect of two naturally occurring compounds, monomeric epicatechin and curcumin, on beta cell respiration.

Protocol

1. Cell Culture

1. Culture INS-1 derived 832/13 beta cells in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 10 mM HEPES, 2 mM Glutamine, 1 mM Sodium Pyruvate, and 0.05 mM 2-mercaptoethanol[9,10,11,12,13].
2. Remove 832/13 cells from a T75 flask using 2 mL of 0.25% trypsin, incubating at 37 °C for 10 min. Neutralize trypsin by adding 8 mL of complete RPMI 1640 media.
3. Count cells using a hemocytometer and dilute 100 µL of the cell volume from step 1.2 in 900 µL of PBS.
4. Plate 832/13 cells at a density of 2 x 10^6 cells/mL in a 6 well dish.
5. Culture cells for 48 h in a humidified incubator at 37 °C and 5% CO_2 before beginning respiration experiments.

2. Preparation of Cells for High-resolution Respirometry

1. **Treating 832/13 cells with cocoa derived epicatechin monomer.**
    1. Culture 832/13 cells for 24 h after plating in a humidified incubator at 37 °C and 5% CO_2.
    2. Change media 24 h after plating, and treat 832/13 cells with vehicle control (3 wells) or 100 nM cocoa monomer (3 wells), followed by culture for an additional 24 h (48 h total) in a humidified incubator at 37 °C and 5% CO_2.
    3. Wash 832/13 cells in 1x Low Glucose Secretion Assay Buffer (SAB) for 5 min. Aspirate buffer, and incubate 832/13 cells in 1x Low Glucose SAB for 3 h, changing 1x Low Glucose SAB every hour.
        1. Make 10x SAB with 33.32 g of NaCl, 1.73 g of KCl, 0.82 g of KH_2PO_4, 0.7 g of $MgSO_4$ and add H_2O to a final volume of 500 mL. Sterile filter the final solution.
        2. Make 1x Low Glucose SAB with 10 mL of 10x SAB, 100 µL of 45% glucose, 2 mL of 1 M HEPES, 1 mL of 0.25M $CaCl_2$, 0.57 mL of 35% BSA solution, 0.21 g of $NaHCO_3$ and add H_2O to a final volume of 100 mL. Sterile filter the final solution.

2. **Treating 832/13 cells with curcumin.**
    1. Culture 832/13 cells for 48 h after plating in a humidified incubator at 37 °C and 5% CO_2.
    2. Wash 832/13 cells in 1x Low Glucose SAB for 5 min. Aspirate buffer, and incubate 832/13 cells in 1x Low Glucose SAB for 3 h, changing 1x Low Glucose SAB every hour.
    3. After 2.5 h of the 3 h incubation in 1x Low Glucose SAB, aspirate buffer and incubate cells in 1x Low Glucose SAB with vehicle control or 40 µM curcumin for 30 min. Following incubation, aspirate the buffer.

3. **Harvesting cells with trypsin for high-resolution respirometry**
    1. After the 3 h incubation in 1x Low Glucose SAB, remove the cells from the plate with 250 µL of 0.25% trypsin per well.
    2. Combine the cells in trypsin from 3 wells treated with vehicle control or compound of interest with 4 mL of SAB in a 15 mL conical tube.
    3. Count cells using a hemocytometer and dilute 100 µL of the cell volume from step 2.3.2 in 900 µL of PBS.
    4. Dilute the appropriate number of cells in 1x Low Glucose SAB to make 3 mL at the appropriate concentration for each chamber. The data demonstrates that a concentration of 1 x 10^6 cells/mL is the most effective.

4. **Harvesting cells with mechanical dissociation for high-resolution respirometry**
    1. After the 3 h incubation in 1x Low Glucose SAB, add 1 mL of 1x Low Glucose SAB to each well and gently blow cells off the plate with mechanical dissociation by pipetting with a 1000 µL pipette tip.
    2. Combine the cells from 3 wells treated with vehicle control or compound of interest in a total of 3 mL SAB in a 15 mL conical tube for respiration.

3. High-resolution Respirometry

1. **Preparation of the high-resolution respirometer.**
    1. Turn on the high-resolution respirometer and connect to the desktop by launching the respirometer analysis program.
    2. Aspirate 70% ethanol from both chambers. Soak these chambers in 70% ethanol for a minimum of 45 min.
    3. Wash the chambers twice with 70% ethanol, aspirating after each step.
    4. Wash the chambers three times with ddH_2O, aspirating after each step.
    5. Rinse the chamber plungers in ddH_2O. This cleans off residual ethanol from the plungers and from the titanium port.

2. **Calibration of polarographic oxygen sensors.**
    1. Add 2.4 mL of 1x Low Glucose SAB buffer to each oxygraph chamber, stirring the buffer continuously using magnetic stir bars in the chamber at 750 rpm and 37 °C with a data-recording interval at 2.0 s by pushing the F7 button and opening the tab labeled "Systems". Push plungers all the way in, and then retract to the wrench aeration setting. Let the machine equilibrate for a minimum of 1 hour until stable oxygen flux is obtained.
    2. Set the machine at 37 °C for the duration of the experiment. Set polarization voltage to 800 mV, with a gain of 2 by pushing the F7 button and opening the tab labeled "Oxygen, O_2". Equilibrate the oxygen concentration of the SAB buffer for at least 30 min, while the change in oxygen concentration is stable (less than 2 pmol/(s*mL)).

3. Following oxygen concentration stabilization, select a region where the change of oxygen concentration is stable to establish background measurement of change in oxygen concentration.
    1. Select a region by pushing the shift key, left clicking on the mouse and dragging the mouse across the selected region. Click on the letter associated with the selected region and change to "R1" for each trace, corresponding with each of the two chambers.
    2. Double click on the "O_2 Calibration" box in the bottom left and right corners of the screen, click the "select mark" button for the "air calibration" as R1 and then select "calibrate and copy to clipboard" for both chambers.

3. **Evaluation of respiration of 832/13 beta cells prepared in steps 2.1.5 or 2.2.5.**
    1. Load 2.4 mL of sample in each chamber (one chamber with control vehicle treated cells, one chamber with compound treated cells) in 1x Low Glucose SAB. Retain 0.5 mL of cell sample for protein quantitation by BCA (Bicinchoninic acid) assay if using the mechanical dissociation approach (freeze at -20 °C for measurement later).
        1. Push plunger in all the way and aspirate the residual volume. Stir the cells continuously throughout the experiment at 750 rpm and 37 °C for all subsequent steps. Make a mark by clicking F4 and labeling as "cells" when samples are loaded.
        2. Measure samples for 30 min. After signal stabilization, select a region of the change in oxygen concentration corresponding to the low glucose conditions (2.5 mM glucose) as described in 3.2.3.
    2. After signal stabilization is reached, add 12.5 µL of a 45% sterile glucose solution (16.7 mM final concentration) into each chamber through the titanium loading port using a syringe. Make a mark measured "Glucose" as described in 3.3.1 when treatment is added. Let signal stabilize and record cellular respiration until a stable oxygen flux is achieved. Select this region of the change in oxygen concentration, as described in 3.2.3. This is the 16.7 mM Glucose reading, and corresponds with stimulatory conditions[7].
    3. After signal stabilization is reached add 1 µL of 5mM oligomycin A (2.5 µM final concentration) into each chamber through the loading port. Make a mark measured "OligoA" as described in 3.3.1 when treatment is added. Let signal stabilize and record cellular respiration until a stable oxygen flux is achieved. Select this area of the curve as described in 3.2.3. Oligomycin A inhibits ATP synthase and thus the only oxygen flux occurring is via leak of electrons and not oxidative phosphorylation[7].
    4. After signal stabilization is reached add 1 mM FCCP in 1 µL increments until a maximum respiration rate is established. This represents maximal uncoupled respiration. Between 3-4 µL FCCP is sufficient (1.5-2.0 µM final concentration) to induce maximal uncoupled respiration of INS-1 832/13 cells. Make a mark labeled "FCCP" as described in 3.3.1 when treatment is added. Let signal stabilize and record cellular respiration until a stable oxygen flux is achieved. Select this area of the curve as described in 3.2.3. FCCP is an uncoupling agent. This allows us to measure uncoupled respiration[7].
    5. After signal stabilization is reached add 1 µL of 5 mM Antimycin A (2.5 µM final concentration) into each chamber through the loading port. Make a mark labeled "AntiA" as described in 3.3.1 when treatment is added. Let signal stabilize and record cellular respiration until a stable oxygen flux is achieved. Select this area of the curve as described in 3.2.3.
    Note: Antimycin A binds cytochrome C reductase, inhibits ubiquinone oxidation, and blocks all respiration. This allows us to completely stop respiration[7].

4. **Measuring 832/13 beta cells for protein concentration and respiration normalization.**
    1. Using the 0.5 mL of sample retained at loading, measure protein sample using the BCA method[13].
    2. Run each sample in triplicate, without dilution, following the manufacturer's instructions.

5. **832/13 beta cell respiration calculations from trypsinized cells**.
    1. Enter the number of cells per mL use in the assay by pushing the F3 button of the respirometer analysis program. Change the units to cells/mL, enter the cellular concentration, and change medium to SAB.
    2. Select background readings to normalize the data by selecting and entering into the O_2 Calibration form as described in 3.2.3.
    3. Make selections of readings from 2.5 mM Glucose, 16.7 mM Glucose, Oligomycin A, FCCP and Antimycin A as described in 3.3.1.
    4. Within the respirometer analysis program, export the readings for each chamber after entering protein concentration and selecting the appropriate average values for each treatment during the respiration measurement. This is done by clicking F2 and then pushing the "copy to clipboard function". This exports the data for use in other analysis programs. Use the "O_2 slope neg." values for calculations.
    5. Compile data for 3-5 independent runs of vehicle treated controls and natural compounds treated cells to determine the effect on intact cellular respiration of INS-1 832/13 beta cells.

6. **832/13 beta cell respiration calculations from mechanically dissociated cells**.
    1. Enter the protein calculations from the BCA assay by pushing the F3 button of the respirometer analysis program. Change the units to mg, enter the BCA concentration, and change medium to SAB.
    2. Select background readings to normalize the data by selecting and entering into the O_2 Calibration form as described in 3.2.3.
    3. Make selections of readings from 2.5 mM Glucose, 16.7 mM Glucose, Oligomycin A, FCCP and Antimycin A as described in 3.3.1.
    4. Within the respirometer analysis program, export the readings for each chamber after entering protein concentration and selecting the appropriate average values for each treatment during the respiration measurement. This is done by clicking F2 and then pushing the "copy to clipboard function". This exports the data for use in other analysis programs. Use the "O_2 slope neg." values for calculations.
    5. Compile data for 3-5 independent runs of vehicle treated controls and natural compounds treated cells to determine the effect on intact cellular respiration of INS-1 832/13 beta cells.

Representative Results

INS-1 832/13 beta cells that are prepared and harvested as described in the protocol will demonstrate modulation in oxygen consumption based on the various chemical interventions (**Figure 1A**). An increase in respiration will be observed when the glucose concentration is increased to 16.7 mM Glucose (**Figure 1B**). Respiration will decrease when the intact cells are treated with Oligomycin A. This respiration is known as LEAK, which is defined as the basal, nonphosphorylating respiratory state. Maximal respiration will be achieved when the beta cells are treated with the

uncoupling agent FCCP, which is defined as ETS respiration, or electron transport system respiration. Finally, respiration will drop to zero when treated with Antimycin A, labeled as ROX (residual oxygen consumption) which refers to the non-respiratory cellular oxygen consumption.

Differences in cell number will significantly change the quality of the data obtained from this technique. Glucose-induce respiration was measured at 0.5, 1, and 2 x 10^6 cells/mL (**Figure 2A-2C**). Using cell counts and BCA protein assays, protein concentrations corresponding to the three tested cell concentrations were calculated (**Figure 2D**). Measurements using 0.5 x 10^6 cells/mL failed to demonstrate glucose-stimulated respiration (**Figure 2A**), and only the ETS associated increased respiration was shown to be significant. This suggests that the low cell number results in a low signal to noise ratio that confounds the results. Similarly, measurements at 2 x 10^6 cells/mL also resulted in significant variability, as indicated by statistical significance only being reached for the ETS measurements (**Figure 2C**). This is presumably due to the need to re-oxygenate the chambers multiple times during the assay, which resulted in greater variability from sample to sample. These data demonstrate that concentrations of 1 x 10^6 cells/mL result in significant glucose-stimulated respiration, LEAK and ETS measurements, which are necessary for respiration analyses (**Figure 2B** and **2E**).

Two methods for removing the cells for respiration are presented in this protocol. Using trypsin to remove cells is effective when measurements are completed at 37 °C. Respiration measurements done in the absence or presence of curcumin (**Figure 3A**) demonstrates maintenance of glucose-stimulated respiration, LEAK and ETS respiration. Comparison of curcumin treated cells to untreated cells demonstrates no change to any of these respiratory parameters (**Figure 3B-C**). Respiration measurements done in the absence or presence of cocoa epicatechin monomer (**Figure 3D**) also demonstrates maintenance of glucose-stimulated respiration, LEAK and ETS respiration. Comparison of cocoa epicatechin monomer treated cells to untreated cells demonstrates increased respiration at 2.5 mM and 16.7 mM Glucose, as well as in LEAK and ETS respiration (**Figure 3E-F**). These data demonstrate that while curcumin does not enhance 832/13 beta cell respiration, cocoa epicatechin monomer does (**Figure 3G**). Curcumin has been shown to enhance insulin secretion through inhibiting phosphodiesterase activity[14]. Our data suggest that curcumin does not enhance insulin secretion through modulating mitochondrial function. We have already demonstrated that culture in the presence of cocoa epicatechin monomers induces expression of various components of the mitochondrial electron transport chain[5]. We also observed increased respiration in the LEAK state (induced by treatment with oligomycin) and with ETS (electron transport system, induced by FCCP). The use of this tool suggests a number of potential targets for future studies.

As an alternative to trypsin removal of cells, mechanical dissociation may also be performed using the presented washing technique when completed at 37 °C. Respiration measurements were done in the absence or presence of curcumin or cocoa epicatechin monomer (**Figure 4A-C**). These data demonstrate that the trends observed with trypsin preparation of the cells are maintained, however the sensitivity appears to be diminished. While control and cocoa epicatechin monomer treated cells maintained the glucose-stimulated, LEAK and ETS respiration, cells treated with curcumin only retained the glucose-stimulated respiration due to the increased sample-to-sample variability. While the trends observed with trypsin cell preparations were maintained (**Figure 4D-F**), the significance decreased in all parameters, presumably to the greater sample-to-sample variability introduced by the mechanical dissociation and the normalization to protein concentration rather than cell number.

These data demonstrate that this protocol can be used to measure respiration of intact INS-1 832/13 beta cells. Furthermore, our protocol suggests an optimum cell number for accurate measurements and a preferred method for preparing the cells to maximize the signal to noise ratio.

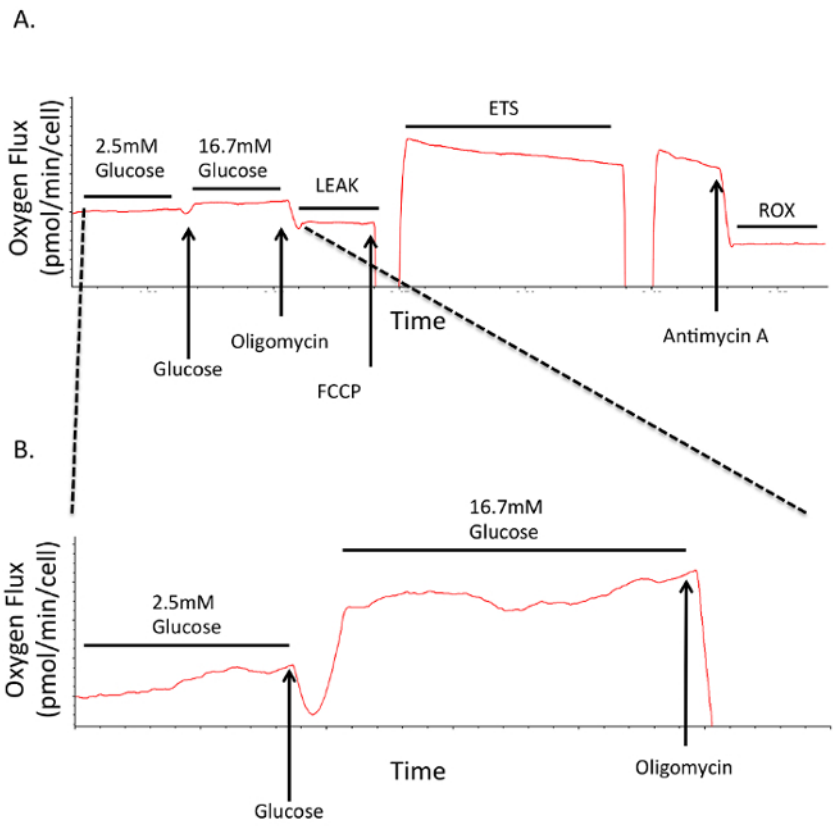

Figure 1: Representative respiration curve of intact 832/13 beta cells. Intact 832/13 beta cells are measured for respiration in (**Figure 1A**) 2.5 mM Glucose, 16.7 mM Glucose, 2.5 µM Oligomycin, 2 µM FCCP (Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone) and 2.5 µM Antimycin A. An expansion of the 16.7 mM Glucose section (**Figure 1B**) is presented to demonstrate the increase in glucose-stimulated respiration. Cells were measured at a concentration of 1 x 10^6 cells/mL. LEAK refers to the basal, nonphosphorylating respiratory state, ETS refers to the uncoupled respiratory capacity of the electron transport system, and ROX refers to the residual oxygen consumption after mitochondrial respiration is inhibited. Please click here to view a larger version of this figure.

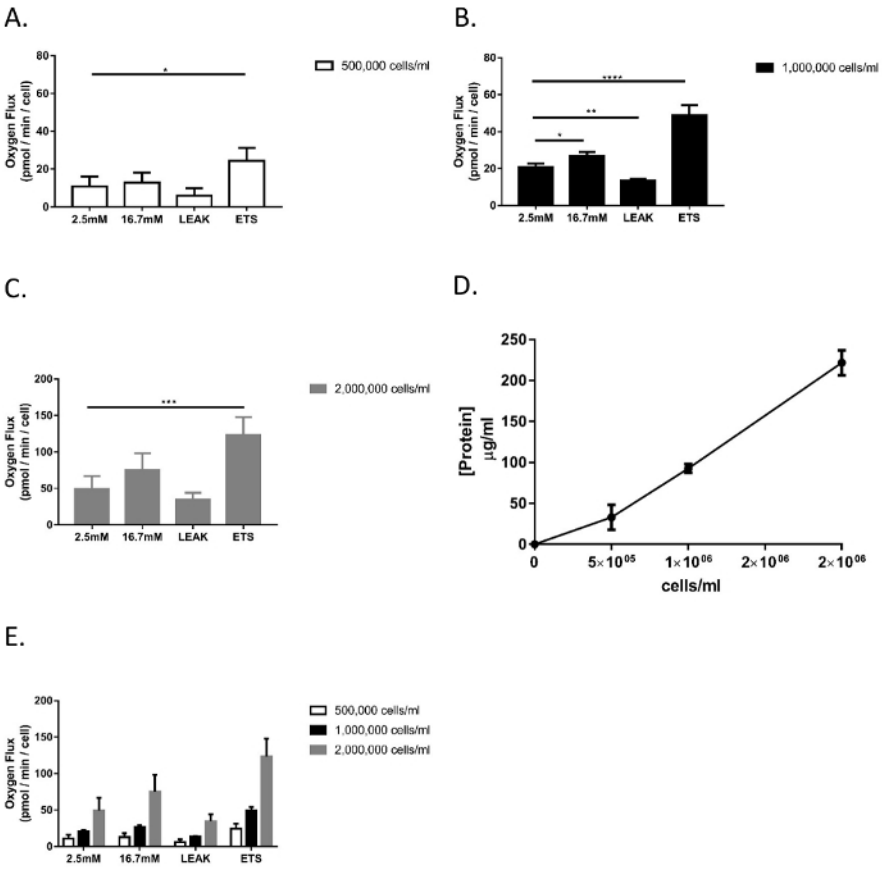

Figure 2: Determination of number of 832/13 beta cells necessary for glucose-induced respiration measurements. Intact 832/13 beta cells are measured for respiration at concentrations of (**A**) 0.5 x 10^6 cells/mL (**B**) 1 x 10^6 cells/mL, or (C) 2 x 10^6 cells/mL. (**D**) Protein concentrations that correspond with 0.5 x 10^6 cells/mL, 1 x 10^6 cells/mL, and 2 x 10^6 cells/mL. (**E**) Comparison of respiration at 0.5 x 10^6 cells/mL, 1 x 10^6 cells/mL, and 2 x 10^6 cells/mL. Respiration was measured under 2.5 mM Glucose, 16.7 mM Glucose, 2.5 µM Oligomycin, 2 µM FCCP and 2.5 µM Antimycin A conditions. Respiration with 2.5 and 16.7 mM glucose demonstrate the glucose-stimulated respiration response of the beta cells. Respiration after oligomycin demonstrates the LEAK respiration (basal, nonphosphorylating respiratory state). Respiration after FCCP (Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone) treatment demonstrates (uncoupled respiratory capacity of the electron transport system, ETS). n = 6 independent runs. Data is presented as average ± SEM. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001. Please click here to view a larger version of this figure.

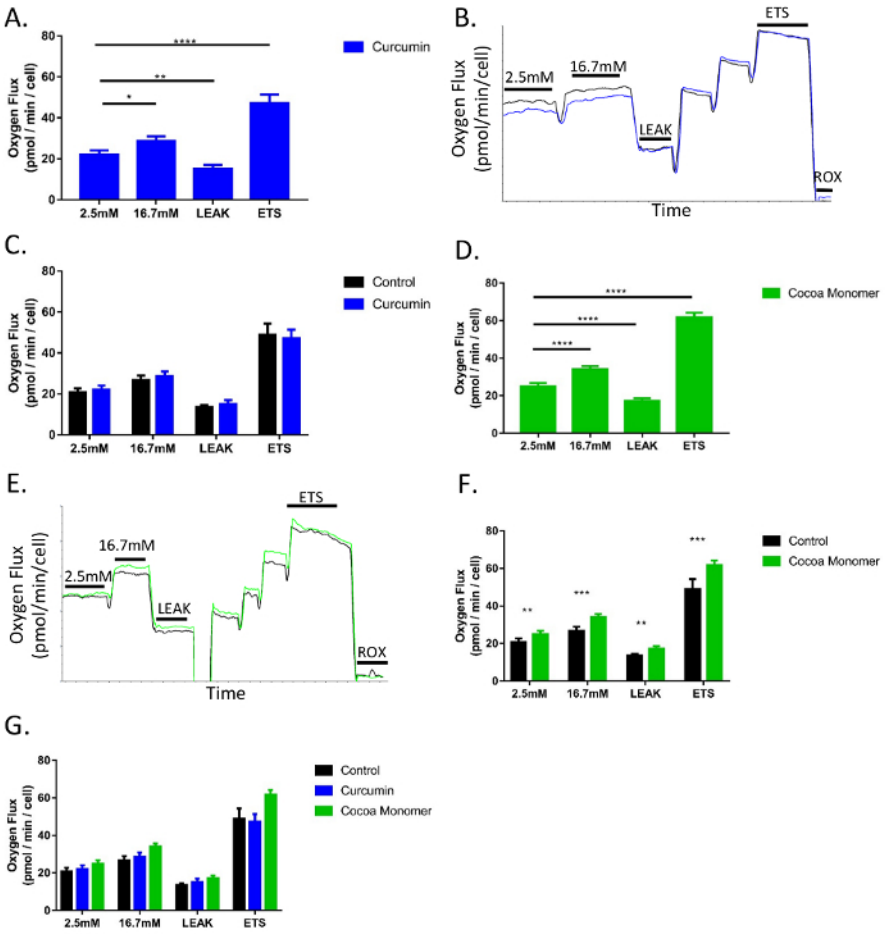

Figure 3: Respiration measurements of 832/13 beta cells after release using trypsin. 832/13 beta cells were cultured in the presence or absence of monomeric cocoa epicatechin or curcumin and respiration was measured after releasing cells using trypsin. Respiration was measured under 2.5 mM Glucose, 16.7 mM Glucose, 2.5 µM Oligomycin, 2 µM FCCP and 2.5 µM Antimycin A conditions. Respiration with 2.5 and 16.7mM glucose demonstrate glucose-stimulated respiration. Respiration after oligomycin demonstrates the LEAK respiration (basal, nonphosphorylating respiratory state). Respiration after FCCP (Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone) treatment demonstrates (uncoupled respiratory capacity of the electron transport system, ETS). Respiration after antimycin A treatment demonstrates residual oxygen consumption (ROX). (**A**) Treatment of 832/13 cells with curcumin maintains the appropriate changes in respiration due to the treatments. (**B**) Representative trace and (**C**) average of data for cells treated with curcumin demonstrates no change to 832/13 beta cell respiration. (**D**) Treatment of 832/13 cells with cocoa epicatechin monomer maintains the appropriate changes in respiration due to the treatments. (**E**) Representative trace and (**F**) average of data for cocoa epicatechin monomer treated cells demonstrates increased respiration in all parameters after cocoa epicatechin monomer treatment. (**G**) All three parameters are presented together for comparison. n = 6 independent runs. Data is presented as average ± SEM. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001. Please click here to view a larger version of this figure.

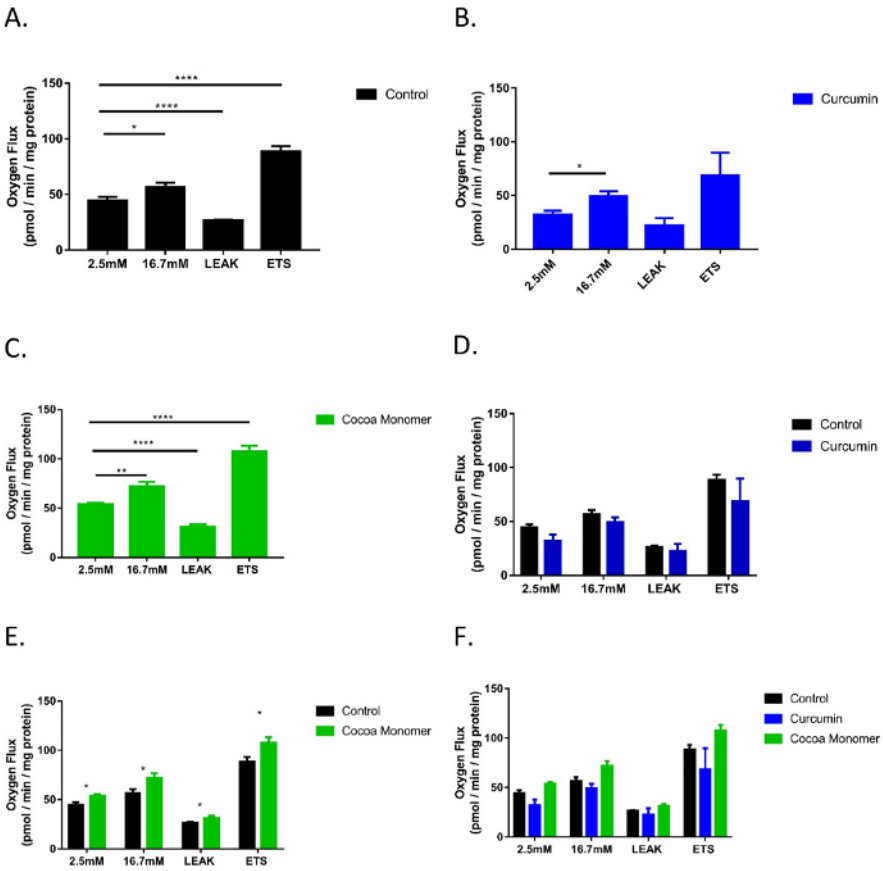

Figure 4: Respiration measurements of 832/13 beta cells after release using mechanical dissociation. 832/13 beta cells were cultured in the presence or absence of monomeric cocoa epicatechin or curcumin and respiration was measured after releasing cells using mechanical dissociation. Respiration was measured under 2.5 mM Glucose, 16.7 mM Glucose, 2.5 µM Oligomycin, 2 µM FCCP and 2.5 µM Antimycin A conditions. Respiration with 2.5 and 16.7 mM glucose demonstrate the glucose response of the beta cells. Respiration after oligomycin demonstrates the LEAK respiration (basal, nonphosphorylating respiratory state). Respiration after FCCP (Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone) treatment demonstrates (uncoupled respiratory capacity of the electron transport system, ETS). Average of data for cells (**A**) untreated cells or cells treated with (**B**) curcumin or (**C**) cocoa epicatechin monomer demonstrate that mechanical dissociation maintains glucose-stimulated respiration in all treatments, while LEAK and ETS significance are lost only in curcumin treated cells. Comparison of untreated cells to (**D**) curcumin or (**E**) cocoa epicatechin monomer demonstrates increased respiration in all parameters with cocoa epicatechin monomer with no significant change to respiration with curcumin. (**F**) All three parameters are presented together for comparison. n = 6 independent runs. Data is presented as average ± SEM. *p <0.05, **p <0.01, ****p <0.0001. Please click here to view a larger version of this figure.

Discussion

The objective of this protocol is to use high-resolution respirometry to measure respiratory rates in intact pancreatic beta cells. This method allows the measurement of the beta cell response to increased glucose levels. The protocol also allows for the pretreatment with various compounds, as demonstrated in this protocol with the naturally occurring monomeric epicatechin or curcumin[4,5]. Treatment with various other compounds could be used in this protocol, with pretreatment (as shown here) or by treating within the respiratory chambers with minimal modifications to the base protocol.

There are various ways by which this method could be modified. Other beta cell lines could be used; however, the cell number and SAB wash times will need to be determined experimentally. Primary rodent and human islets could also be used; however, the number of cells or islet equivalents will also need to be determined experimentally. At least one study has measured islet oxygen consumption using high-resolution respirometry[15]. This study required 400 islets per reaction, but did not measure glucose induced respiration changes. Given that primary tissue is frequently used with high-resolution respirometers[16,17], intact islets could be used after experimental troubleshooting. However, given the large number of islets required for this assay, other methods of measuring oxygen consumption may be better suited for measurements of primary islet respiration.

There are a few critical steps with the presented protocol. First, having an adequate number of cells is critical. Our studies show that 832/13 beta cells at a concentration of 1 x 10^6 cells/mL is sufficient for glucose-stimulated respiration measurements. Measurements using 0.5 x 10^6 cells/mL demonstrated low glucose-stimulated respiration and a low signal to noise ratio. Furthermore, measurements using 2 x 10^6 cells/mL resulted in high sample to sample variability, and rapid cellular use of oxygen which required chamber re-oxygenate and frequently resulted in rapid cell death. The appropriate number of cells should be determined experimentally for the given cell line in order to troubleshoot this critical step.

A second critical step is preparing the beta cells to respond to elevated glucose levels. The RPMI 1640 culture media has high glucose levels. The cells must be moved from the high glucose to a low glucose culture. A 3 h incubation in 1x Low Glucose SAB is sufficient for the beta cells to have a significant response to the addition of ~16.7 mM glucose. Wash periods of less than 3 h result in variable responses to the addition of glucose. Troubleshooting glucose response problems can be addressed by increasing the length of SAB wash time.

A third critical step is the method for preparing the cells for measurements. Our data demonstrates that while trypsin and mechanical dissociation can both be used, trypsin released cells result in more consistent readings and less sample-to-sample variability. The trend observed with both methods was maintained, however the strength of the data was greater with the cells prepared using trypsin. While other methods, such as cell scraping, may be used our data suggest that the use of trypsin will result in the most reproducible results with less sample-to-sample variability[18,19].

Limitations of this approach include the following. First, with only two treatment chambers, this is not a high throughput screening method. Each run of two samples takes between 3-4 h, including the time to prepare the machine. Second, a relatively large concentration of cells is required for the assay, given the ~2.2 mL of sample (or ~2.2 x 10^6 cells) needed per chamber. Due to the amount of sample required for the assay, the use of this tool and protocol for primary islets is limited unless a large supply is available. The previous studies that have used islets for respiration in this technique used ~400 islets/chamber[15]. This would require, roughly, islets from two mice for each chamber[20]. Third because permeabilized cells are not being used, the ability to determine the effect of the treatment on individual electron chain components by using compounds that are not cell permeable is not available.

There are a number of significant advantages of this protocol with respect to existing methods. First, this protocol allows for the stepwise titration of various membrane permeable compounds. This allow for the accurate determination of necessary concentrations. Second, use of intact beta cells allows us to query the effect on cytosolic and mitochondrial glucose metabolism[7]. This allows us to observe phenotypes indicative of changes to the glycolytic pathway, an observation that is lost when using permeabilized cells. Third, the intact cells allow us to use the response to elevated glucose as a metric of beta cell function, which is lost in permeabilized variants of this assay. Finally, this protocol allows for the addition of various and multiple compounds to query the effect of compounds on beta cell respiration, a luxury that is not present with other tools that measure respiration.

Future applications of this method include the ability to measure parameters such as ATP, calcium, and H_2O_2 levels concurrent with respiration measurements. This method also allows for measurement of respiration in the presence of other fuel sources, such as fatty acids or ketones. Finally, with some modifications, measuring secreted insulin levels concomitant with respiration measurements may also be possible.

Disclosures

The authors have nothing to disclose.

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