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Rates of insulin secretion in INS-1 cells are enhanced by coupling to anaplerosis and Kreb's cycle flux independent of ATP synthesis

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ABSTRACT

Mechanistic models of glucose stimulated insulin secretion (GSIS) established in minimal media in vitro, may not accurately describe the complexity of coupling metabolism with insulin secretion that occurs in vivo. As a first approximation, we have evaluated metabolic pathways in a typical growth media, DMEM as a surrogate in vivo medium, for comparison to metabolic fluxes observed under the typical experimental conditions using the simple salt-buffer of KRB. Changes in metabolism in response to glucose and amino acids and coupling to insulin secretion were measured in INS-1 832/13 cells. Media effects on mitochondrial function and the coupling efficiency of oxidative phosphorylation were determined by fluorometrically measured oxygen consumption rates (OCRs) combined with ^{31}P NMR measured rates of ATP synthesis. Substrate preferences and pathways into the TCA cycle, and the synthesis of mitochondrial 2nd messengers by anaplerosis were determined by ^{13}C NMR isotopomer analysis of the fate of [U-^{13}C] glucose metabolism.

Despite similar incremental increases in insulin secretion, the changes of OCR in response to increasing glucose from 2.5 to 15 mM were blunted in DMEM relative to KRB. Basal and stimulated rates of insulin secretion rates were consistently higher in DMEM, while ATP synthesis rates were identical in both DMEM and KRB, suggesting greater mitochondrial uncoupling in DMEM. The relative rates of anaplerosis, and hence synthesis and export of 2nd messengers from the mitochondria were found to be similar in DMEM to those in KRB. And, the correlation of total PC flux with insulin secretion rates in DMEM was found to be congruous with the correlation in KRB. Together, these results suggest that signaling mechanisms associated with both TCA cycle flux and with anaplerotic flux, but not ATP production, may be responsible for the enhanced rates of insulin secretion in more complex, and physiologically-relevant media.

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1. Introduction

Mechanistic models of glucose stimulated insulin secretion (GSIS) established in minimal media in vitro, may not accurately describe the complexity of coupling metabolism with insulin secretion that occurs in vivo. Central to the mechanism of nutrient-stimulated insulin secretion is that increases in mitochondrial oxidative phosphorylation lead to increases in cytosolic ATP concentrations that initiate the cascade of events leading to insulin exocytosis [1]. As a first approximation, one would predict that changes in the rate of oxidative phosphorylation should be directly proportional to changes in oxygen consumption rates (OCRs) and insulin secretion. Surprisingly though, the correlation between OCR (and presumably oxidative phosphorylation) and insulin secretion has been shown to depend upon the complexity of the media [2]. In mouse βHC9 cells, Papas and Jerema [2] observed the change in insulin secretion was proportional to the change in OCR, when the cells were stimulated with 15 mM glucose in minimal media (PBS). These results are in agreement with the predictions of the role of mitochondrial ATP production leading to closure of the K_{ATP} channel to increase Ca^{2+} and trigger insulin exocytosis. However, despite a 2-fold increase in insulin secretion in response to 15 mM glucose, there was no observed change in OCR, and presumably oxidative phosphorylation, in more nutrient-complex Dubelco's Minimal Eagle Media (DMEM) buffer.

Possible explanations to account for this anomalous disassociation between insulin secretion rates and OCR include media-associated changes in (1) mitochondrial coupling of ATP synthesis with flux of the electron transport chain and oxygen consumption, (2) anaplerosis-dependant increases in the export of mitochondria 2nd messengers, or possibly (3) the induction of other cytosolic signaling intermediates or pathways that are coupled to insulin secretion.

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In order to shed light on which of these potential mechanisms may account for the disassociation of nutrient-stimulated insulin secretion from OCR and possibly oxidative phosphorylation, we evaluated the correlation of fuel-stimulated insulin secretion with (1) the putative uncoupling of mitochondrial oxidative phosphorylation, and (2) the rates of mitochondrial anaplerosis. We used ^{31}P NMR to measure rates of ATP synthesis and to measure the concentrations of ATP and intracellular Pi that occurred upon stimulating insulin secretion by increasing glucose from 2.5 to 15 mM [3,4]. OCRs were measured under identical conditions fluorometrically in a sealed, stirred bioreactor [2,5]. To assess the possibility that mitochondrial anaplerotic flux and the synthesis of putative 2nd messengers may provide an explanation for the disconnect between oxygen consumption and insulin secretion in the complex media, we used a ^{13}C-isotopomer approach to determine pathways of anaplerosis in response to the secretagogues of glucose, glutamine and leucine [6,7]. The studies described herein evaluated these correlations using the rat insulinoma cell line INS-1 832/13 [8] in the physiologically-complex DMEM for comparison to results obtained in KRB.

2. Materials and methods

2.1. Cell culture

Initial stocks of clonal INS-1 832/13 cells, overexpressing the human insulin gene, were obtained from the laboratory of Dr. Christopher B. Newgard (Duke University School of Medicine) [8]. INS-1 cells were cultured as monolayers in RPMI-1640 as previously described [6].

Basal metabolic parameters were measured at 2.5 mM glucose in order to maintain stable intracellular ATP concentrations while avoiding nutrient-starvation. In preliminary NMR experiments, we observed that culture of the cells at 0 mM glucose in KRB led to a rapid fall in ATP concentrations and loss of cell viability. The addition of glucose to these ATP-deficient cells then led to a burst of ATP production to re-establish more physiologically-sustainable concentrations of ATP. In contrast, with 2.5 mM glucose, ATP concentrations remained stable and cell viability was maintained during a 24-h perfusion.

3. ^{13}C-Isotopomer studies of anaplerotic pathways

Flux of metabolites via the anaplerotic pathways of pyruvate carboxylase (PC) or glutamate dehydrogenase (GDH), relative to the tricarboxylic acid (TCA) cycle, were determined by analysis and modeling of the ^{13}C-isotopomer pattern in glutamate as previously described [6,7]. Briefly, basal conditions were established with a 2 h pre-incubation in DMEM with a sub-stimulatory concentration of glucose (2.5 mM). The cells were then washed with PBS, and then incubated for an additional 2 h in DMEM with [U-^{13}C] glucose (Cambridge Isotope Laboratories, Miamisburg, OH, 99% ^{13}C, 2.5 or 15 mM), alone or supplemented with 4 mM glutamine and 10 mM leucine. Media aliquots were taken and placed on ice for insulin analysis at 0, 1, and 2 h. At the end of the 2 h isotopic labeling period, the cells were quenched and extracted for ^{13}C NMR analysis. Relative ^{13}C enrichment and isotopomer distribution of glutamate was determined by ^{13}C NMR spectroscopy with an AVANCE 500-MHz spectrometer (Bruker Instruments, Inc., Billerica, MA) [6].

3.1. Metabolite analysis

Total protein was measured spectrophotometrically, based on the method of Bradford (Bio-Rad). Insulin was determined by ELISA for rat insulin (100% cross-reactivity to human insulin, ALPCO).

3.2. Oxidative phosphorylation: ^{31}P NMR measured rates of ATP synthesis and fluorometric measured rates of oxygen consumption

ATP synthesis rates of alginate-encapsulated INS-1 cells were determined in real time using a ^{31}P NMR saturation transfer pulse sequence, as previously described [3,4]. Briefly, INS-1 cells were perifused with KRB or DMEM (flow = 1.0 ml/min, 37 °C) in the bioreactor in the bore of the AVANCE-500 NMR spectrometer. After a pre-equilibration period with 2.5 mM glucose, ^{31}P NMR spectra and saturation transfer experiments were acquired during step changes in secretagogues, glucose, and glutamine plus leucine. ^{31}P NMR spectra were continuously collected in 20-min experiments, with 3–4 measurements collected for each substrate level.

ATP synthesis rates were calculated from the intracellular Pi concentration and the ATP synthesis rate constant [3,4]. Basal intracellular Pi (13.0 ± 1.2 nmol/mg-protein) was measured using tandem mass spectrometry. The ATP synthesis rate constant was calculated from the longitudinal relaxation time of intracellular Pi, and the change in the intracellular Pi signal when the terminal phosphate of ATP is saturated compared to a control spectra (i.e., the saturation pulse is symmetrically offset from Pi resonance).

3.3. Oxygen consumption rates

Oxygen consumption rates of freshly trypsinized cells or encapsulated cells were measured using a Fiber Optic Oxygen Monitor (Model 210, Instech Laboratories, Plymouth Meeting, PA) [5]. A 250 μl chamber was loaded with $\sim 2 \times 10^6$ cells, or ~5 beads and changes in the oxygen concentration were monitored under buffer conditions and substrate additions identical to ^{31}P NMR experiments for measurement of ATP synthesis rates.

3.4. Viability of entrapped cells as determined by OCR

Freshly trypsinized cells were used for this work and the OCR was measured in three different media: DMEM, RPMI (as used for INS-1 cell culture) and G0-PBS. The fractional viability as measured with the Guava PCA (live/dead staining) was 97 ± 2% $n = 5$ and was not significantly different in any of the three media. In addition viability measurements were conducted before and after the OCR measurement (by removing cells from the OCR chamber) and were not significantly different. OCR in glucose and glutamine-free PBS is 49 ± 7% ($n = 4$) of that measured in RPMI and DMEM, and are in excellent agreement with measurements made with alginate-entrapped INS-1 cells. We also conducted a measurement with pyruvate stimulation (2 mM) in G0-PBS and observed an increase of more than 100%, again in very close agreement with previous measurements in KRB and PBS with entrapped cells. These data suggest that the metabolic responsiveness of freshly trypsinized INS-1 cells is essentially the same as entrapped, overnight-cultured INS-1 cells used for our ^{31}P NMR studies of ATP synthesis.

3.5. Statistical analyses

All data are reported as means ± SEM. Unpaired two-tailed student's *t*-tests were used for comparisons between groups. Differences were considered statistically significant at $P < 0.05$.

4. Results and discussion

4.1. Media effects on insulin secretion rates, oxygen consumption rates (OCRs), and rates of ATP synthesis

As the original studies demonstrating the discrepancy between of correlation of OCR and insulin secretion rates in DMEM vs. KRB

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