Pulses of Glucose and Combined Glutamine/Serum Stimulate TCA Cycle Flux in Cultured βHC9 Insulinomas

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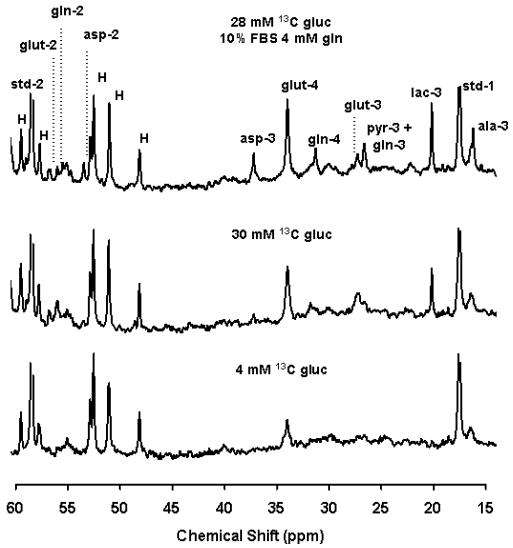
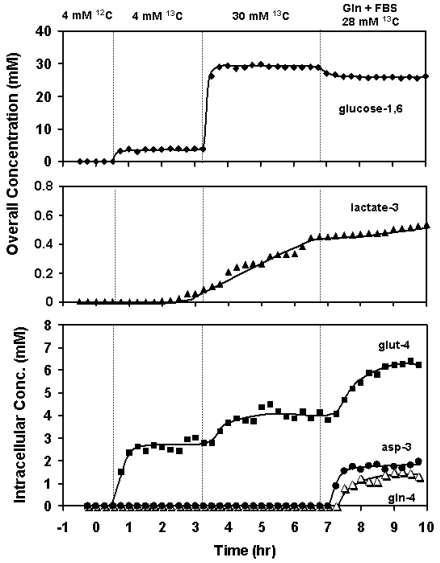
Introduction: Insulinomas are immortalized insulin secreting cells that could potentially be used in a bio-artificial pancreas (1,2). For both insulinomas and normal pancreatic beta cells, insulin secretion is stimulated by elevated glucose, some amino acids and lipids (3). However, unlike other extracellular signals such as hormones that bind to a receptor, these compounds must be metabolized before their levels can be sensed. The exact mechanism that connects metabolism of these compounds and insulin secretion has not been elucidated. A better understanding of this mechanism could facilitate genetic engineering of insulinomas to improve their functioning in an artificial pancreas. Since catabolism of these substrates produces cellular energy, ATP has been hypothesized to be a regulator of insulin secretion (3). However, previous NMR studies suggest that this is unlikely, although ADP may play a role (3). In this work, the effects of various substrates on the flux of ^{13}C from [1,6-^{13}C] glucose into metabolic intermediates was examined. Intracellular metabolite levels were detected non-invasively with NMR in cells that were immobilized on non-porous microcarriers. This method was used rather than standard gel entrapment to avoid oxygen diffusion limitations that can obscure true metabolic rates (4).

Methods: βHC9 insulinomas were grown in thin layers on microcarriers that were pre-coated with ProNectin® F (Solohill, Ann Arbor, MI), a recombinant fibronectin. This polypeptide was necessary to enable the cells to attach and spread on the microcarriers, since they are not strongly adherent. The microcarriers were used in a tightly packed bed (they are essentially incompressible) and continuously perfused at 5 ml/min with oxygenated medium in a 20-mm NMR tube. Polarographic probes were used to measure the inlet and outlet dissolved oxygen concentrations of the perfusate (5). pH was monitored with a probe in the NMR tube outlet line. The pH and dissolved oxygen levels were controlled by adjusting the ratio of N_2, O_2, and CO_2 flowing through a gas exchange device. Temperature was monitored with a thermocouple on the NMR tube inlet line and maintained at 37 ± 0.2°C with a microstate controller. All probes were interfaced to a laptop computer though a PCMCIA analog to digital converter controlled with LabVIEW (National Instruments). NMR spectra were acquired with a 9.4 Tesla system (Varian Inova). A 20-mm liquids probe was used for both ^{31}P and ^{13}C spectroscopy. ^{13}C spectra were acquired with 60 ° pulses, a repetition time 1.2 s, 8,192 points and a spectral width of 25,000 Hz. Bi-level WALTZ16 decoupling was used to enhance the S/N by NOE and to eliminate proton-carbon splittings. Free induction decays were zero filled to 16,384 points and apodized with exponential multiplication (10 Hz). Cells were grown in DMEM with 25 mM glucose and 15% serum. During ^{13}C NMR spectroscopy, cells were initially perfused with modified Hanks balanced salt solution (HBSS, 4.6 mM KCl, 0.40 mM KH_2PO_4, 137 mM NaCl, 0.31 mM Na_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, 0.25% bovine serum albumin (Fraction V), 1% fetal bovine serum (FBS), pH=7.4) that contained 4 mM unlabeled glucose. Subsequently, the perfusate was replaced with a solution of the same composition, but with [1,6-$^{13}C_2$] glucose instead of unlabeled glucose. Further changes are described in the results section.

Results: NTP measurements indicated that the total cell number in the NMR detectable volume was 8 x 10^8, based on a cell diameter of 12 μm (determined by light microscopy). With the introduction of [1,6-$^{13}C_2$] glucose, C-4 glutamate labeling was detected almost immediately and the resonance grew for approximately 45 minutes (See figures below). A small amount of labeled C-3 lactate was also observed. The volume of medium recirculating through the packed bed was relatively large (110 ml) so that the glucose level was not significantly depleted during the experiment. After C-4 glutamate labeling reached an approximate steady state, the [1,6-$^{13}C_2$] glucose concentration was increased from 4 mM to 30 mM, without any other changes. This increased the oxygen consumption rate from 0.061 mmol/hr to 0.076 mmol/hr. Strong labeling was observed in C-4 of glutamate and weak labeling was observed in C-2 and C-3. Label was also detected in C-3 lactate and C-3 alanine. Glutamate is in rapid equilibrium with the α-ketoglutarate in the TCA cycle (6). The increased labeling in glutamate and the increased oxygen consumption rate are indicative of increased flux into the TCA cycle. To determine if the TCA cycle flux could be further stimulated, 4 mM unlabeled glutamine and 10% FBS were added to the HBSS containing 30 mM [1,6-$^{13}C_2$] glucose. With these additions, enhanced labeling was detected in C-2, C-3 and C-4 of glutamate, C-2 and C-3 of aspartate, and C-3 of lactate and alanine. Glutamine was also labeled in C-2, C-3, and C-4. Examination of the perfusate alone with ^{13}C NMR indicated that all of these compounds were intracellular. The rate of formation of labeled lactate, which is predominantly extracellular, slowed markedly with the addition of glutamine and FBS.

Discussion: The results indicate that increasing the glucose level from 4 to 30 mM markedly increased the flux into the TCA cycle. This is consistent with the belief that the flux through primary pathways is integrally connected with insulin secretion. In addition, this flux was further increased by the combined pulse of glutamine with FBS. This result was expected since glutamine is known to stimulate insulin secretion in the presence of leucine. Further, the reduced lactate formation rate indicated that with glutamine and FBS, glucose is used more aerobically. The steady state labeling levels in glutamate could be used to calculate pathway fluxes if the size of the intracellular pool of glutamate was known (7). The unsteady state data could also be used to calculate fluxes, but improved S/N for C-3 and C-2 glutamate would be necessary. Microcarrier coverage with βHC9 cells was only ~50% and cells that are more strongly adherent could give substantially higher S/N. We have already identified a cell line derived from the INS-1 insulinoma that grows well on microcarriers, which will be used for our future work.. Nevertheless, the results of this work demonstrate that NMR spectroscopy can be used to monitor intracellular dynamics in insulinomas.





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