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INSULIN RELEASE AND ADENINE NUCLEOTIDE CHANGES IN INS-1 CELLS IN RESPONSE TO A GLUCOSE CHALLENGE: CREATINE LOADING IN SMALL CELL BIOREACTORS STUDIED BY ^{31}P-NMR SPECTROSCOPY

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ABSTRACT

INSULIN RELEASE AND ADENINE NUCLEOTIDE CHANGES IN INS-1 CELLS IN RESPONSE TO A GLUCOSE CHALLENGE: CREATINE LOADING IN SMALL CELL BIOREACTORS STUDIED BY ^{31}P-NMR SPECTROSCOPY

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Glucose-induced insulin release is proposed to involve transmission of an energetic signal from β-cell mitochondria to the membrane K^{+}_{ATP} channel. Closing of the K_{ATP} channel through either increased [ATP] or decreased [ADP] has been implicated in membrane depolarization and 1st phase insulin secretion. 2nd phase insulin secretion reaches a maximum 45 minutes following a glucose challenge and is dependent on membrane depolarization and continued glucose metabolism Thus glucose leads to altered energetics and induces both phases of insulin secretion. We test this hypothesis via ^{31}P-NMR analysis of creatine loaded INS-1 cells packaged into bioreactors. Transition from 2.0 to 16.7 mM glucose caused Pi to decrease and PCr to increase, substantially amplifying the phosphorylation potential. There were no changes in the ATP peak, but calculations based on the creatine kinase equilibrium showed a significant decrease in ADP. Additionally, intracellular acidification was induced by a glucose challenge. The energetic changes were completely reversible and occurred in both creatine and non-creatine loaded cells. Kinetically these cellular changes reached a maximum 45 minutes after the glucose challenge. Taken together, these results suggest that the 1st and 2nd phases of glucose induced insulin release may both be regulated by decreases in cytosolic ADP.

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SYMBOLS/ABBREVIATIONS

Cr ---------- Creatine
PCr---------- Phosphocreatine
Pi---------- Inorganic Phosphate
ATP---------- Adenosine Triphosphate
ADP ---------- Adenosine Diphosphate
^{31}P-NMR---------- ^{31}Phosphorouse Nuclear Magnetic Resonance
K^+_{ATP} channel ---------- Inward Rectified, ATP-Sensitive Potassium Channel
Mg^{2+} ---------- Magnesium
Ca^{2+} ---------- Calcium
RR Pool ---------- Readily releasable pool of insulin granules
IR Pool ---------- Immediately releasable pool of insulin granules
$[ATP]_s$ ---------- Submembrane [ATP]
$[ATP]_i$ ---------- Intracellular [ATP]
NADH---------- Nicotinamide Adenine Dinucleotide
$FADH_2$ ---------- Flavin Adenine Dinucleotide
NBF ---------- Nucleotide Binding Fold
C° ---------- Degrees Celsius

INTRODUCTION

Glucose homeostasis is a vital physiological mechanism ensuring adequate cellular energy supply. Blood glucose levels are maintained within a narrow range by coordinated action of several tissues: skeletal muscle, liver, adipose, and pancreatic islets of Langerhans [1]. Muscle, liver, and adipose tissue serve as the major glucose sinks, storing glucose in the form of glycogen and triglycerides, while pancreatic islets serve as glucose sensors and secrete hormones to control glucose uptake and mobilization.

In response to a carbohydrate-rich meal, an increase in plasma glucose is detected by the islets. Islet α-cells respond by decreasing the secretion of glucagon, a hormone which elevates plasma glucose by stimulating glycogen breakdown and gluconeogenesis. In contrast islet β-cells respond by increasing insulin secretion into the blood stream. Insulin, the only blood glucose lowering hormone, functions by stimulating glucose uptake and utilization.

Circulating insulin is bound by tyrosine kinase receptors located on the cell membranes of skeletal muscle, hepatocytes, and adipocytes. Sufficient insulin signaling promotes glucose uptake and storage while preventing glucose mobilization (especially in the liver). In terms of glucose uptake, adipocytes and myocytes have insulin sensitive glucose transporters (GLUT4). Insulin signaling increases the number of GLUT4 transporters in the cell membrane by promoting the translocation of the receptor from the cytoplasm to the cell surface thereby enhancing glucose uptake. In terms of glucose usage and storage, myocytes and hepatocytes respond to insulin by upregulating enzymes important for glucose metabolism (phosphofructokinase, pyruvate dehydrogenase) and glycogen synthesis (glucokinase/hexokinase, glycogen synthase) while the key enzymes

for glycogenolysis and gluconeogenesis (glycogen phosphorylase, glucose-6-phosphatase) are downregulated. Additionally, insulin signaling in the liver and adipocytes promotes the storage of glucose metabolites as triglycerides by stimulating enzymes associated with lipogenesis (acetyl CoA carboxylase, fatty acid synthase) while inhibiting enzymes important for the breakdown of fatty acids and triglycerides (carnitine acyl-transferase I, and hormone sensitive lipase). As a result of these coordinated mechanisms, blood glucose levels are tightly regulated.

The importance of glucose homeostasis is strikingly illustrated in patients with type I and type II diabetes mellitus. Type I insulin dependent diabetes occurs when pancreatic β-cells are selectively destroyed by an autoimmune reaction, and thus the ability to secrete insulin and lower blood glucose is completely lost. Before Frederick Banting and Charles Best discovered insulin in 1922, type I diabetics died within a short time of developing the disease. Type II non-insulin dependent diabetes develops from a combination of peripheral insulin resistance and a progressive inability of the β- cell to secrete adequate amounts of insulin. Elevated plasma glucose due to inadequate uptake is further exacerbated by pathological glucose secretion by the insulin insensitive diabetic liver. In both insulin controlled type I diabetics and type II diabetics, improper glucose control promotes hyperglycemia and thereby contributes to a number of serious health problems including cardiovascular disease, atherosclerosis, neuropathy, nephropathy, and retinopathy induced blindness. Although the causes of type I and type II diabetes are unclear, implicit in both disorders is the vital importance of proper glucose induced insulin secretion.

Glucose Induced Insulin Secretion

Glucose-induced insulin release from pancreatic ß cells exposed to an experimental square wave of stimulation occurs in two phases. First phase insulin secretion is dependent on β-cell electrical activity and peaks around 5 minutes after a glucose challenge in isolated rat islets [2]. This first phase is normally induced by glucose metabolism, but can be experimentally reproduced in the absence of glucose by any manipulation that elevates intracellular Ca^{2+}. Second phase insulin secretion accounts for most of the physiological release and involves a more gradual, sustained rise in insulin exocytosis that peaks 30-45 minutes after stimulation. This second phase is also dependent on sustained β-cell depolarization but is additionally mediated by processes that are absolutely dependent on glucose metabolism.

Several non-mutually exclusive mechanisms have been proposed to explain the differences between first and second phase insulin secretion, most notably the "signal-limited model" and the "storage-limited model." [3]. Although the "signal limited model" will not be discussed at length, its proponents hypothesize that either a biphasic signal or the sum of stimulatory and inhibitory signals with different time-courses underlie the two phases of insulin secretion. The "storage-limited model" proposes that three distinct pools of insulin granules exist within the β- cell: immediately releasable (IR) pool, readily releasable (RR) pool, and reserve pool [2]. The IR pool is thought to be docked at the cell membrane and primed for exocytosis while the RR pool is morphologically docked but requires priming before secretion. Additionally there is a large reserve pool (>95% of total insulin) that is neither docked nor primed. In this model, first phase insulin secretion is attributed to exocytosis of the IR pool while the rate

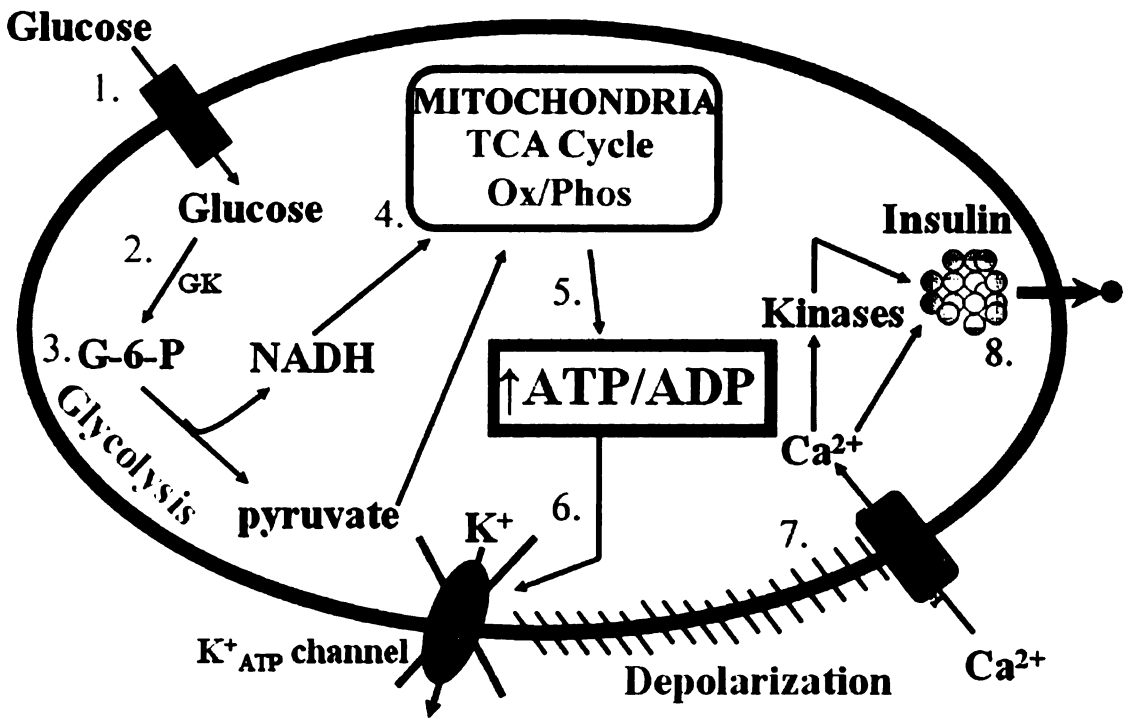

Figure 1: Classic Understanding of Glucose Induced Insulin Release
1) Glucose entry via GLUT2 2) Glucokinase (GK) converts glucose to glucose-6-phosphate (G-6-P) 3) Glycolysis utilizes G-6-P to produce NADH and pyruvate 4) Mitochondrial TCA cycle and oxidative phosphorylation (Ox/Phos) to produce ATP 5) Increase in ATP/ADP ratio which 6) closes the inward rectified K^+_{ATP} channel. 7) Depolarization 8) Activation of the voltage-gated Ca^{2+} channel. 9) $[Ca^{2+}]_i$ activates secretory mechanisms for insulin granule release.

limiting component of second phase is the replenishment of the IR pool via ATP, Ca^{2+}, and temperature dependent priming of the RR pool. Within the "storage-limited model" it is controversial whether a single signal or different signals mediate exocytosis of the IR pool and priming of the RR pool respectively. Support for the storage limited model comes from experiments demonstrating release of a small amount of insulin under conditions that raise $[Ca^{2+}]_i$ [3] [4] and morphological observations of a Ca^{2+} independent increase in insulin granules near the plasma membrane following glucose stimulation [5]. Additional validation of the "storage limited model" will require better understanding of the signals and mechanisms regulating docking, priming, and exocytosis as well as a clear understanding of the relationship of these mechanisms to 1^{st} and 2^{nd} phase insulin secretion.

β-cells are unique among excitable cells in their dependence on glucose metabolism for stimulus secretion coupling. Although the metabolic events that differentiate first and second phase insulin secretion are controversial, it is generally agreed that the signals underlying both phases directly involve enhanced glucose metabolism that alters cellular energetics, β-cell electrical activity, and intracellular Ca^{2+} levels. More specifically, following a rise in plasma sugar, glucose transport across the β- cell plasma membrane occurs via the high capacity glucose transporter, GLUT2 (Figure 1). A sufficient rise in cytoplasmic glucose is sensed by the rate limiting enzyme glucokinase whereby glucose is phosphorylated and then rapidly metabolized via glycolysis. Glycolytic products are used as substrates for the TCA cycle and mitochondrial oxidative phosphorylation, thus changing the energetic state of the cell. It has been hypothesized that this energetic change is manifested as an alteration in

cytosolic adenine nucleotide levels capable of closing adenosine triphosphate sensitive potassium channels (K^+_{ATP} channels) [6]. The closing of these inward-rectified K^+ channels, which normally leak K^+ to maintain the membrane potential, depolarizes the cell and stimulates the opening of voltage-gated Ca^{2+} channels. [7] The subsequent rise in intracellular Ca^{2+} is essential for insulin secretion [8].

The rise in intracellular Ca^{2+}, mediated by closing of membrane K^+_{ATP} channels, is widely regarded as the triggering signal for 1st phase insulin secretion. After a glucose challenge $[Ca^{2+}]_i$ rises just before first phase insulin secretion and remains elevated in the presence of stimulatory glucose [3]. Increasing $[Ca^{2+}]_i$ in the absence of glucose via nonphysiological depolarization (high K^+ or sulphonylureas) induces first phase insulin secretion. In the absence of glucose, 2nd phase insulin secretion does not occur. Further, insulin secretion that resembles 2nd phase can be induced by glucose metabolism when K^+_{ATP} channels are held open and $[Ca^{2+}]_i$ is artificially elevated [9]. If K^+_{ATP} channels are held open and $[Ca^{2+}]_i$ is not elevated, glucose metabolism does not induce insulin secretion [10]. Thus, glucose metabolism induces a K^+_{ATP} dependent rise in intracellular Ca^{2+} that triggers 1st phase insulin secretion while signals generated by glucose metabolism combine with triggering $[Ca^{2+}]_i$ to amplify insulin secretion during the 2nd phase. The exact mechanism of Ca^{2+} induced exocytosis is unclear. As in other exocytotic cells, β- cells have an association of N-ethylmaleimide sensitive fusion protein attachment protein receptor (SNARE) proteins that are important for exocytosis. Of these proteins, synaptotagmin V and IX appear to be directly involved in Ca^{2+} dependent stimulation of exocytosis. The mechanisms responsible for priming of insulin granules are even less well-understood. There does, however, appear to be an ATP dependent step

distal to the rise in $[Ca^{2+}]_i$ [11]. Additionally, acidification of insulin granules may be necessary for priming and exocytosis [12].

While much has been discovered about glucose induced insulin release during the last thirty years, the nature and temporal control of the signals coupling glucose oxidation to physiological 1st and 2nd phase insulin release remain poorly defined. Mitochondrially determined changes in adenine nucleotides are widely thought to gate the K^+_{ATP} channel and control the subsequent Ca^{2+} influx and 1st phase insulin secretion. Despite widespread support, changes in adenine nucleotides have not been clearly and consistently demonstrated in insulin secreting cells. The metabolic signals mediating 2nd phase insulin secretion are more controversial but have been proposed to include malonyl CoA, long chain acyl-CoAs, citrate, diacyl glycerol, protein kinase A (PKA), protein kinase C (PKC), phospholipase A_2, phosphoinositides, glutamate, and adenine or guanine nucleotides [2]. Adenine nucleotides are an attractive regulator that would provide a single, energetically based mechanism controlling 1st and 2nd phase insulin secretion.

Mitochondria and K^+_{ATP} Channels

Despite the confusion surrounding glucose induced insulin secretion, it is clear that mitochondria and K^+_{ATP} channels are essential components of stimulus secretion coupling. Mitochondria are proposed to operate as secretagogue (i.e. glucose) sensors, controlling metabolism and the energetic status of the β-cell. In this model K^+_{ATP} channels serve as sensors of the cellular energetic state, altering β-cell electrical activity in response to mitochondrial signals. Evidence for the importance of mitochondria and

K^+_{ATP} channels in stimulus secretion coupling is derived from a variety of studies using activators, inhibitors, and genetic manipulation of the two vital components.

Mitochondria and Glucose Metabolism

Mitochondria contain all the enzymes necessary for the TCA cycle and oxidative phosphorylation promoting the transfer of energy from the carbon bonds of glucose to the terminal phosphate of ATP. Specifically this process begins with the glycolytic breakdown of glucose in the cytoplasm, which generates nicotinamide adenine dinucleotide (NADH) in addition to ATP and pyruvate. NADH is transported into the mitochondria via the α-glycerolphosphate [13] and malate/aspartate shuttles [14]. Once transported inside the mitochondria, NADH contributes electrons to complex I of the electron transport chain, a group of four protein complexes (I-IV) located within the inner mitochondrial membrane. β-cells contain very low levels of lactate dehydrogenase [15] so most of the pyruvate produced by glycolysis is transported into the mitochondria where it is converted to either acetyl-CoA or oxaloacetate (OAA) by pyruvate dehydrogenase or pyruvate carboxylase. Acetyl-CoA and OAA can then be shuttled into the TCA cycle to generate the reducing equivalent flavin adenine dinucleotide ($FADH_2$), which subsequently contributes electrons for oxidative phosphorylation via complex II. The complexes of the electron transport chain, by a series of oxidation and reduction reactions, use the energy of electrons from NADH and $FADH_2$ to transport protons out of the inner membrane and produce an electrochemical potential across the inner mitochondrial membrane. The energy of this proton gradient is used by the F_1-ATP synthase to generate ATP from ADP and Pi. Uncoupling protein 2 (UCP-2) is a

transmembrane channel that, when inserted into the inner mitochondrial membrane, dissipates the proton gradient and prevents oxidative ATP production. ATP produced by the mitochondria is finally transported to the cytoplasm by the adenine nucleotide translocator (ANT) for use by cellular processes.

Glycolysis and the TCA cycle thus upregulate mitochondrial oxidative phosphorylation by increasing substrate availability (NADH, $FADH_2$, ADP). It has also been reported that mitochondrial oxidative phosphorylation is regulated by intracellular Ca^{2+} [16, 17]. However, the availability of ADP is the principal energetic determinant of cellular respiration, a correlate measurement of mitochondrial oxidative phosphorylation [18]. Increases in ADP stimulate oxidative phosphorylation to promote ATP synthesis. It has been proposed that the first step in glycolysis, the rate limiting conversion of glucose to glucose-6-phosphate by glucokinase using ATP, increases ADP and stimulates mitochondrial respiration [19] [20]. Overall, upregulation of mitochondrial oxidative metabolism in response to a glucose challenge is thought to produce the relevant energetic signals (increase in ATP and decrease in ADP), controlling insulin release through the gating of K^+_{ATP} channels. Nevertheless the nature of the energetic signal is unclear.

Glucose induced insulin secretion is associated with an increase in mitochondrial respiration that reaches a new steady state maximum approximately 45 minutes after a sustained glucose challenge [21]. Modulation of mitochondrial function has significant effects on insulin secretion. Overexpression of UCP-2 depresses glucose induced insulin secretion, likely by uncoupling glucose induced electron transport from ATP production, thus keeping the ATP:ADP ratio low. Experimental inhibition of oxidative

phosphorylation markedly inhibits glucose induced insulin secretion [22] [23] while mice with targeted knockout of mitochondria do not exhibit glucose induced insulin secretion [24].

The remarkable depression of glucose induced insulin secretion following pharmacological or genetic inhibition of the mitochondria is viewed as evidence that mitochondrial oxidative phosphorylation of glycolytic products generates the key energetic signal (increase in ATP:ADP ratio) for insulin secretion [25]. Indeed more than 95% of β-cell ATP is produced by the mitochondria [26], and it was recently demonstrated that insulin secretion initiated by four different secretagogues was tightly correlated with mitochondrial metabolism as opposed to glycolytic flux [27]. Unfortunately most experiments have measured either the input variable (oxygen consumption, glucose concentration/metabolism) or the output variable (insulin secretion, K^+_{ATP} channel activity, $[Ca^{2+}]_i$) without measuring or calculating the signal that may relate mitochondrial glucose metabolism to downstream K^+_{ATP} channel activity and insulin secretion. Using ^{31}P-NMR we are able to measure several relevant signals (ATP, PCr, Pi, ADP) connecting mitochondrial metabolism with K^+_{ATP} channel gating. Characterization of the K^+_{ATP} channel supports our proposal that mitochondrial produced signals are major regulators of glucose induced insulin secretion.

K^+_{ATP} Channel

The K^+_{ATP} channel isoform found in pancreatic β-cells was reconstituted by Inagaki et al in 1995 and is composed of a high affinity sulfonylurea receptor (SUR1) and an inward rectified potassium channel ($K_{IR}6.2$) assembled as a tetramer (SUR1/ $K_{IR}6.2)_4$

on the cell surface [28]. $K_{IR}6.2$ forms the pore of the channel, and binding of ATP (or ADP) by any of the four subunits promotes closing of the channel [29] [30]. SUR1 is homologous to members of the ATP-binding cassette (ABC) superfamily containing two cytosolic nucleotide-binding folds (NBF1 and NBF2), each of which is capable of binding ATP, MgATP and MgADP [31] to open the channel.

The K^+_{ATP} channels is proposed as one of the critical regulators of pancreatic β-cell membrane potential, leaking K^+ during periods of low glucose to maintain the negative membrane potential [32]. Ashcroft et al in 1984 demonstrated that a glucose challenge inhibited a K^+ current in rat islets [6]. Closure of K^+_{ATP} channels via signals derived from glucose metabolism is thus thought to depolarize the cell and promote Ca^{2+} entry through voltage sensitive Ca^{2+} channels. Pharmacologically, opening islet K^+_{ATP} channels with diazoxide prevents changes in β-cell electrical activity and inhibits glucose induced insulin secretion [33]. This discovery explains the drug's ability to induce experimental diabetes in lab animals [34, 35]. In contrast, artificially closing K^+_{ATP} channels with a class of drugs called sulfonylureas, that bind the SUR1 subunit, induces hyperinsulinemia and is a common treatment for insulin deficient type II diabetes [36]. More recently, genetically modified mice lacking K^+_{ATP} channels have been found to have serious disruptions in glucose induced insulin secretion [37].

Finally, K^+_{ATP} channel gating has been demonstrated to be directly related to mitochondrial function. Various inhibitors of mitochondrial function stabilize the K^+_{ATP} channel open state and prevent glucose induced K^+_{ATP} closure. Misler et al (1989) demonstrated that sodium azide, an inhibitor of the terminal enzyme of mitochondrial electron transport (cytochrome oxidase/complex III), activates K^+_{ATP} channels by

decreasing the ATP:ADP ratio [38]. Cyanide [39] and oligomycin [40] also open K^+_{ATP} channels by decreasing the ATP:ADP ratio via inhibition of mitochondrial NADH dehydrogenase (complex I) and the F_1-ATPase respectively. Finally, the hyperglycemic effects of bongkrekic acid were attributed to its ability to prevent glucose induced K^+_{ATP} channel closure by inhibiting the mitochondrial adenine nucleotide translocator (ANT) in mouse islets and preventing an increase in the ATP:ADP ratio[41]. In contrast agents that activate electron transport by supplying reducing equivalents directly to cytochrome C oxidase (complex III) are able to close of K^+_{ATP} channels [42]. Duchen et al demonstrated that tetramethyl p-phenylenediamine (TMPD) promoted an increase in the inner mitochondrial membrane potential, a rise in $[Ca^{2+}]_i$, and an increase in whole cell K^+_{ATP} currents of mouse islets . TMPD reduces cytochrome C oxidase, thus directly activating ATP synthesis in the absence of fuel metabolism [43, 44], and did not have a further effect when applied concurrently with a glucose challenge. Taken together this data demonstrates an implicit relationship between mitochondrial metabolism and K^+_{ATP} channel activity. Despite the anecdotal evidence for this relationship, the exact nature of the glucose induced mitochondrial signals and the mechanism by which these signals regulate the K^+_{ATP} channel remains unclear.

Mitochondrial Energetic Signals and Regulation of the K^+_{ATP} channel

It was initially proposed that ATP was the intracellular messenger linking a glucose challenge to alteration of the K^+_{ATP} open state [45]. Application of exogenous ATP to a patch clamp was effective in closing conducting K^+_{ATP} channels. However, subsequent investigation revealed that the K^+_{ATP}-channel's affinity for ATP (I_{50}~15 μM)

was much higher than the cellular concentration of ATP (3-5 mM) which would render all K^+_{ATP} channels inactive [28, 46].

Shortly after the discovery that ATP could close K^+_{ATP} channels, ADP was demonstrated to antagonize the effects of ATP. The ability of ADP to activate K^+_{ATP}-channels closed by ATP was initially demonstrated by two separate groups. The experiments of Dunne and Peterson (1986), found that 100-500 μM ADP was able to open patch clamped channels from RINm5F cells closed by 500 μM ATP [47]. Misler et al (1986) presented data showing that 80-1200 μM ADP reopened K^+_{ATP}-channels from rat β-cells closed by 400 μM ATP [39]. Hopkins et al (1992) first suggested that K^+_{ATP}-channels contain more than one adenine nucleotide binding site regulating channel gating [48]. Cloning and in-vitro reconstitution of the K^+_{ATP}-channel components confirmed and expanded their prediction, providing great insight into the molecular/metabolic regulation of channel gating and its relation to insulin release.

Manipulation of the channel structure has helped to elucidate the possible role of the K^+_{ATP} channel as a sensor of the cellular energetic status. When $K_{IR}6.2$ is expressed without the SUR1 subunit, the subsequent channels are chronically closed and insensitive to the cellular metabolic state (ATP/ADP) [29]. SUR1 is therefore thought to be the regulatory subunit of the K^+_{ATP} channel responsible for reversing ATP-induced closure. As previously mentioned SUR1 contains two cytosolic nucleotide-binding folds (NBF's), each of which binds ATP, MgATP and MgADP [31]. Expression of SUR1 with an ATP insensitive $K_{IR}6.2$ pore subunit, resulted in a channel that was activated by MgADP and MgATP [49]. Photoaffinity labeling of the nucleotide binding sites with 8-azido-[^{32}P]ATP as well as mutagenesis of NBF1 and NBF2 demonstrated that NBF1 binds ATP

with high affinity in a Mg^{2+}-independent manner while NBF2 was found to be a low affinity Mg^{2+}-dependent ADP and ATP binding site [50] [51]. Since ADP stimulates K^+_{ATP} channel activity only in the presence of Mg^{2+} [52], and mutating NBF2 produces K^+_{ATP} channels insensitive to the stimulatory effects of MgADP [53], it was proposed that NBF2 is essential for channel activation. Furthermore, binding of MgADP at NBF2 was shown to stabilize binding of ATP at NBF1 [54]. Ueda et al therefore proposed a model where ATP binding to NBF1, stabilized by MgADP binding to NBF2 holds the K^+_{ATP} channel in an open conformation [54]. A decrease in the intracellular concentration of ADP or an increase in ATP would then promote dissociation of MgADP from NBF2, destabilize the open state, and close the channel. The fact that NBF2 has a similar affinity for MgATP and MgADP together with evidence that both MgADP and MgATP open K^+_{ATP} channels suggests that hydrolysis of MgATP to MgADP may be an important regulator of the K^+_{ATP} channel gating. Indeed, hydrolytic activity has been directly demonstrated in NBF2 of cardiac SUR2A subunits [55] and indirectly demonstrated in NBF2 of the β-cell SUR1 isoform [56]. A mechanism has emerged where MgADP opposes ATP induced channel closure, but the metabolic regulation of each nucleotide by the mitochondria and its direct role in K^+_{ATP} channel gating following a glucose challenge are still unsettled.

Based on structural characterization of the K^+_{ATP} channel and its sensitivity to inhibitory ATP, several mechanisms have been proposed for the regulation K^+_{ATP} channel gating: 1) Submembrane (compartmentalized) concentrations of ATP exist that are low enough to affect channel activity. 2) ADP shifts the sensitivity of K^+_{ATP} channels to physiological ranges of whole cell ATP changes. The ATP:ADP ratio would then be the

relevant metabolic signal. 3) ADP itself is the physiological mediator of K^+_{ATP} channel activity and glucose induced decreases in ADP close the channel and promote insulin secretion. While these proposed mechanisms are not necessarily mutually exclusive (especially #2 and #3), evidence for each will be dealt with separately.

Compartmentation

Proponents of "compartmentation" argue that mechanisms (membrane ATPases, adenylate kinases, creatine kinases) exist to create a localized submembrane concentration of ATP ($[ATP]_s$) around membrane K^+_{ATP} channels that is lower than the bulk cytosolic ATP ($[ATP]_i$) and thus maintains the channel in the open state [57] [58]. Following an influx of glucose and its subsequent metabolism in the β-cell, the "submembrane" concentration of ATP increases, which closes K^+_{ATP} channels and promotes insulin secretion. One of the mechanisms proposed to locally increase $[ATP]_s$ while leaving bulk cytosolic $[ATP]_i$ unaffected is a "phosphocreatine shuttle" [57]. In this model mitochondrial ATP production is buffered by the creatine kinase (CK) enzyme situated in the outer mitochondrial membrane, which serves to increase cytosolic phosphocreatine (PCr). A rise in cytosolic PCr increases the $[ATP]_s$ while decreasing $[ADP]_s$ through a CK associated with membrane K^+_{ATP} channels. The increase in $[ATP]_s$ is theoretically sufficient to close nearby K^+_{ATP} channels. Unfortunately, this proposal is virtually impossible to directly demonstrate with current methods. In support of this model is the K^+_{ATP} channel's high affinity for inhibitory ATP in contrast to cytosolic levels and the existence of a CK enzyme associated with the mitochondrial membrane [57]. While there does appear to be a CK associated with the SUR2A subunit of the

cardiac K^+_{ATP} channels isoform, this has not been shown in the β-cell isoform composed of SUR1 subunits [59]. Moreover, muscle ^{31}P experiments in 1994 by McFarland et al argue against the existence of compartmentalized concentrations of PCr or ATP in skeletal muscle [60] while comparative measurements of $[ATP]_s$ and $[ATP]_i$ in β-cells using luciferin targeted to the cytosol, mitochondrial membrane, and plasma membrane demonstrated that $[ATP]_s$ in these compartments was immediately equilibrated with the bulk cytosolic $[ATP]_i$ [61].

Whole Cell Changes in ATP

It has also been suggested that the sensitivity of the β-cell K^+_{ATP} channel to inhibitory ATP is substantially higher when exposed to experimental media solutions in isolated inside-out patches than when located in an unmanipulated β-cell and exposed to the physiological cytoplasm (discussed below). If this is the case, one could hypothesize that fluctuations in intracellular ATP within the physiological range are capable of opening and closing the less sensitive K^+_{ATP} channels. Indeed, several signaling entities normally present in the cytoplasm are proposed to decrease the sensitivity of K^+_{ATP} channels to ATP including phosphoinositides (PPIs), long-chain acyl-CoA esters, and MgADP [2, 62].

Mutational analysis of the K^+_{ATP} channel by Tarasov et al demonstrated a role for MgADP in shifting the ATP sensitivity of $K_{IR}6.2$ to sense changes in ATP in the physiological range [63]. Mutation of $K_{IR}6.2$ that rendered the pore protein less sensitive to ATP while retaining MgADP sensitivity increased whole cell current (open probability of the K^+_{ATP} channel) when expressed in oocytes. Coexpression of the $K_{IR}6.2$ mutation

with a SUR1 mutation that abolished MgADP sensitivity resulted in channels with lower basal currents than the $K_{IR}6.2$ mutation alone, thus demonstrating a role for MgADP in channel activation. In addition, metabolic inhibition (via sodium azide) in cells expressing channels with the double mutation (less ATP sensitive, MgADP insensitive), decreased ATP levels and further activated whole cell currents suggesting that changes in ATP, detected by $K_{IR}6.2$ may be able modulate channel activity. It should be noted that the importance of MgADP sensing was also demonstrated in these experiments. Although the authors suggested that changes in MgADP were not sufficient to mediate channel activity, this was not demonstrated and therefore cannot be excluded.

The hypothesis that ATP is the physiological mediator of the K^+_{ATP} channel is critically dependent on the assumption that intracellular ATP actually changes when β-cells are exposed to high glucose. While this idea has generally been accepted, experimental data has been confusing at best [64, 65]. A recent nuclear magnetic resonance imaging (NMR) study of insulin secreting β-HC9 cells seemed to suggest that [ATP] was unaffected by glucose [66]. Following a glucose challenge energetic changes in the creatine-loaded cells were reported via PCr and Pi, but careful examination of the NMR spectra showed that the ATP peaks did not change. Apart from this unquantified ^{31}P-NMR study, the current knowledge of adenine nucleotide levels in β-cells is largely based on measurements from acid quenched cell extracts quantified by HPLC or a luciferase assay. Acid quenching is an imprecise process that can result in a significant amount of ATP hydrolysis while the luciferase assay consumes ATP. Quantification of ATP from acid quenched cell extracts also involves comparing samples from a number of different heterogeneous cell populations, an approach with inherent drawbacks. Finally,

neither HPLC or luciferase measurement of cell extracts can distinguish between bound ADP (much larger concentration) and free ADP (physiologically relevant). To address these issues, we developed a ^{31}P-NMR based bioreactor system allowing dynamic measurement (every 8-16 minutes) of ATP in living cells. Use of this system allowed direct evaluation of cytosolic ATP and calculation of free ADP in a single population of cells over time to assess each nucleotides role in K^+_{ATP} channel regulation and glucose induced insulin secretion.

Whole Cell Changes in ADP

While most researchers agree that ADP plays a part in closing K^+_{ATP} channels, confusion still remains whether 1) a decrease in [ADP] is the pertinent metabolic signal or 2) an increases in [ATP] is the direct metabolic signal and ADP simply acts indirectly to lower the sensitivity of the channel to this ATP fluctuation.

The importance of ADP in control of insulin secretion was strikingly demonstrated by the discovery of a human mutation in the putative MgADP binding site of SUR1 (NBF2) that resulted in persistent hyperinsulinemic hypoglycemia of infancy [53]. The analogous mutation was introduced into hamster SUR1 and reconstituted in COSm6 cells. The resultant K^+_{ATP} channels could be opened by a K^+ channel opener (diazoxide), but were unresponsive to the metabolic state of the cells and thereby chronically closed leading to cell depolarization. Further experimentation revealed that the mutant channels, in contrast to their wild type counterparts, were normally responsive to ATP but unresponsive to MgADP. This established the indispensable nature of MgADP regulation of K^+_{ATP} channels, but did not necessarily distinguish between the

role of ADP as the modulator of $K_{IR}6.2$ ATP sensitivity or ADP as the direct metabolic signal.

Several labs support a model where ADP regulates K^+_{ATP} channel gating [67] [68] [55]. In such a model high levels of MgADP are proposed to stabilize the open state of the channel. Decreases in free ADP would therefore increase the dissociation of bound MgADP from NBF2 and promote channel closure. Zingman and colleagues, using inside out patches of cardiac K^+_{ATP} channels, demonstrated that application of PCr and creatine kinase, which promotes the production of ATP from free ADP thereby lowering cytosolic ADP, promoted channel closing. [55]. This channel inhibition was proposed to be caused by MgADP dissociation from NBF2, although an increase in ATP cannot be excluded.

Although several studies have shown that elevated glucose lowers the steady state level of ADP in β-cells [69] [70], and ADP is able to open K^+_{ATP} channels previously closed by ATP [39, 47], little quantitative information is available about glucose-induced changes in free, unbound ADP. Further, the I_{50} for MgADP has never been stringently established, thus preventing the absolute quantification of the physiological concentrations of ADP and ATP necessary to influence the activity of K^+_{ATP} channels during glucose induced insulin release.

Despite the conflicting data surrounding adenine nucleotide changes in β-cells, most researchers within the diabetes field simply refer to the ATP:ADP ratio as the critical regulator of the K^+_{ATP} channel. Hopkins et al (1992) explored the relevance of the ATP:ADP ratio in gating excised K^+_{ATP} channels[48]. They concluded that the ratio, regardless of absolute nucleotide concentrations did not correlate with channel activity.

Instead, nucleotide concentrations within a certain physiological range were important for channel activity. Specifically 250 μM ADP was able to maintain K^+_{ATP} channels 29.5% open in the presence of 1.75 mM ATP. At a higher ATP concentration 500 μM ADP maintained 6% of channel activity in the presence of 3.5 mM ATP. While not ruling out the importance of ATP changes, their data did support a model where ADP changes within the micromolar range may be able to open and close K^+_{ATP} channels in the absence of ATP fluctuations.

Critically important for supporting the role of ADP as the principle regulator of K^+_{ATP} channel gating is demonstration of glucose induced changes in free ADP. Free ADP is maintained at low concentrations (20-150 μM) within cells and is therefore below the detection limit of most assays. Further, as previously discussed, measurements of ADP from cell extracts cannot distinguish between free and bound ADP. Our ^{31}P-NMR system allowed for the quantification of PCr, Pi, and ATP. In conjunction with HPLC measurements of Cr, accurate calculations of free ADP were made using the creatine kinase (CK) and ATPase equilibriums.

Experimental Questions

Many questions remain to be answered concerning the energetic signals coupling mitochondrial glucose metabolism and K^+_{ATP} channel regulated insulin secretion. The purpose of this study was to characterize the energetic changes associated with glucose induced insulin secretion. In so doing, several questions were addressed: 1) How does the energetic status (ATP, ADP, PCr, Cr, Pi) of β-cells change following a glucose challenge? 2) Specifically, how do ATP and ADP change following a glucose challenge?

3) What is the time course of these changes? 4) What is the relationship between these energetic changes and 1st and 2nd phase insulin secretion? 5) What is the relationship between the free energy of ATP and insulin secretion? 6) Can these changes modulate the K^+_{ATP} channel open probability? 6) Could these energetic changes be associated with something other than K^+_{ATP} channel gating?

We address these questions through the development of a bioreactor system that allowed for the characterization and quantification of dynamic energetic changes in creatine and non-creatine loaded insulin secreting cells. Importantly, this approach avoids the potential problems of quantifying nucleotides from acid extracts of several populations of cells artificially quenched at different time points.

Major Experimental Tools

INS-1 Cells

Experimental characterization of glucose induced insulin secretion has traditionally been carried out using primary cultures of mouse or rat pancreatic islets, isolated from the pancreas by partial enzymatic digestion. Islets have the advantage of being a physiologically relevant model and exhibiting a 10-15 fold increase in insulin secretion following a glucose challenge. To their disadvantage, the isolation process for islets is not particularly precise, the isolated cell mass is heterogeneous (β-cells, α-cells, δ-cells, F cells), and they cannot be cultured for longer than 2-3 days, thus preventing significant experimental manipulation. More recently, continuous cultures of glucose responsive cell lines have been established that maintain many of the characteristics of β-cell insulin secretion [71].

In 1992 Asfari and colleagues isolated an insulin secreting cell line (INS-1) from an X-ray induced rat insulinoma [72]. Histologically, INS-1 cells appear similar to native β-cells, retain β-cell surface antigens, and stain positive for insulin. When cultured, the cells adhere to treated plates and can be stably passed more than 80 times. Functionally, INS-1 cells exhibit a 2 to 4-fold glucose induced increase in insulin secretion, which is independent of adherence [73]. This insulin secretion is dependent on K^+_{ATP} closure and depolarization induced Ca^{2+} influx. Compared to other insulin secreting cell lines, INS-1 cells are relatively stable and differentiated, respond to physiological levels of glucose, and synthesize and store greater amounts of insulin [71]. INS-1 cells also contain an enzymatic makeup similar to native β-cells, expressing high levels of pyruvate dehydrogenase and low levels of lactate dehydrogenase to promote oxidative phosphorylation of glucose metabolites [74].

Phosphorous Nuclear Magnetic Resonance Spectroscopy and Cell Bioreactors

Phosphorous nuclear magnetic resonance spectroscopy (^{31}P NMR) is a valuable tool for non-invasive quantification of phosphate containing metabolites (ATP, ADP, PCr, Pi). Measurement of these metabolites enables an assessment of cellular energetics. Early ^{31}P NMR experiments measured the static nucleotide concentrations from cellular extracts. More recently, perifusion systems have been constructed that allow real time, non-invasive nucleotide quantification of isolated muscles [75] and immobilized cell populations [76] [66]. These systems permit the measurement of metabolite changes following various experimental perturbations (muscle stimulation, drug treatment). The threshold level of detection for in-vitro ^{31}P NMR is approximately 500 μM so ADP

(normally < 200 μM) must be calculated based on the ATPase and CK equilibriums. In terms of glucose induced insulin secretion, several real time ^{31}P NMR systems have been developed. Most of these systems involve various methods of encapsulating the cells (agarose beads, alginate-polylysine-alginate (APA) beads, polylysine coated-polystyrene microcarrier beads) to immobilize cells and prevent excessive peak broadening. Current systems require a large number of cells (> $1.5x10^9$) paired with the addition of many spectra to produce adequate signal to noise. We developed a bioreactor whereby a smaller number of cells ($10x10^6$ INS-1 cells) uniformly loaded without beads into a permeable membrane produced higher resolution spectra (8-16 minutes/spectrum). Use of this system in combination with knowledge of the creatine kinase equilibrium allows for the quantification and kinetic characterization of nucleotide changes in creatine and non-creatine loaded INS-1 cells following a glucose challenge.

Creatine Kinase Equilibrium

Creatine kinase (CK) catalyzes the conversion of PCr +ADP $\rightarrow$ Cr + ATP. The enzyme exists in several tissue-specific isoforms: M-CK, B-CK, MB-CK in the cytosol of skeletal muscle, brain tissue, and cardiac muscle respectively [77]. Additionally, there is a mitochondrial isoform of CK (Mi-CK) that localizes to the inner mitochondrial membrane [78]. Significant levels of creatine and phosphocreatine are present in islet-enriched extracts of rat pancreatic β-cells [65, 79]. The B-CK isoform is strongly expressed in salmon islets [80] and mouse β-cells [81] while INS-1 cells express Mi-CK and B-CK[57]. The equilibrium constant for CK is temperature and pH dependent and under physiological conditions favors the synthesis of ATP. PCr and CK in skeletal and

cardiac muscle are thought to function as an energetic buffering system to prevent large fluctuations in adenine nucleotides [82]. More controversial is the proposed role of CK in compartmentalized energy transfer [83] [84]. A role for CK in glucose induced insulin secretion has been suggested [57, 58], but never directly demonstrated. However, increases in PCr have been associated with reciprocal decreases in Cr following nutrient [65] [85] and hormonal [86] stimulation of islets. This data, coupled with the role of CK in buffering adenine nucleotide levels (proposed physiological regulators of the K^+_{ATP} channel) suggests that the creatine kinase equilibrium may be a useful tool for studying insulin secretion.

Overview

In the studies described herein, we have utilized insulin quantification in combination with non-invasive ^{31}P NMR spectroscopy and HPLC analysis of cellular extracts to characterize and quantify the impact of a glucose challenge on the energetic status (ATP, ADP, PCr, Pi, and ΔG_{ATP}) of INS-1 cells. A subset of cells were also creatine loaded to increase the phosphocreatine levels and thereby enhance the signal to noise of the ^{31}P NMR spectra. Our results demonstrate that cellular insulin content and insulin secretion in response to both low and high glucose were both decreased by chronic creatine loading. However, the fold difference in insulin secretion was unchanged. Following a sustained glucose challenge, a series of energetic changes took place whereby inorganic phosphate and creatine decreased, phosphocreatine increased, and ATP levels remained unchanged in both creatine and non-creatine loaded cells. By calculation, free ADP decreased, driving an increase in the free energy of ATP (ΔG_{ATP}).

Although all of these energetic changes were evident during the first measured time point (16 minutes after glucose challenge) they reached their peak approximately 48 minutes after the glucose challenge This time point corresponds to the peak of 2^{nd} phase insulin secretion in rat islets. The time course of energetic changes suggests that a decrease in ADP may regulate both 1^{st} and 2^{nd} phase insulin secretion. Additionally, intracellular acidification was observed that peaked slightly after the energetic changes. This acidification may be an important rate-limiting step for 2^{nd} phase insulin secretion. Finally, all of these changes were demonstrated to be reversible following removal of the glucose challenge.

METHODS AND MATERIALS

Common Cell Culture Methods

Rat insulinoma (INS-1) cells were plated at an initial density of $10x10^6$ cells in T-75 flasks and grown under 5% CO_2 (37°C) with media changes every 48 hr with RPMI-1640 containing 11.1 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES (pH 7.4), 50 μM ß-mercaptoethanol, 10% fetal bovine serum (FBS), and 1% penicillin/streptomyocin [87]. Cells were grown to confluence over a period of 5 days then switched to RPMI-1640 media containing 5.0 mM glucose, 10 mM HEPES, 10% FBS-certified, 1% penicillin/streptomyocin, 50μM ß-mercaptoethanol/sodium pyruvate and either 40 mM creatine (creatine-loading media) or no supplemental creatine (creatine-free media) for a period of 48 hrs. Immediately prior to experiments cells were washed with phosphate-free Krebs Ringer Buffer (NMR KRB) containing 122 mM NaCl, 4.75 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5.0 mM $NaHCO_3$, 30.0 mM HEPES (pH 7.4), and 0.1% BSA equilibrated with 95% O_2 and 5% CO_2 and supplemented with either 2.0 (low glucose) or 16.7 mM glucose (high glucose). An external NMR standard of 3.0 mM phenyl phosphonic acid (PPA) was added to the medium. There was no significant difference in the amount of insulin released from INS-1 cells incubated in NMR KRB media relative to standard KRB assay medium (data not shown).

Nuclear Magnetic Resonance Spectroscopy Studies

Phosphorus NMR of INS-1 cell extracts

To study energetic changes in INS-1 cells in response to changes in glucose concentration, cells were plated into four T-75 flasks at $10x10^6$ cells per flask and subject

to standard culture conditions in either creatine-free (two flasks) or 40 mM creatine-loading (two flasks) media. Phosphorus metabolite changes were measured in response to a glucose challenge as follows: One flask from each group was subjected to low glucose and one to high glucose in phosphate-free KRB for 1.5 hours for creatine-free and creatine-loaded cells (for a total of four flasks). Following exposure to these glucose concentrations, phosphorus metabolites were extracted from plated cells by acid quenching. In brief, harvested cells were exposed to 1.0 mL of ice-cold 0.5 M perchloric acid containing 5 mM EDTA, mechanically harvested from the flasks and sonicated twice for five seconds with a Heat-Systems Microsonicator. Samples were immediately centrifuged at 10,000 g in a pre-cooled SS-34 rotor to precipitate insoluble proteins and membrane fragments. A known volume of the supernatant was placed in a fresh tube and then neutralized (pH 7.0) with an appropriate volume of 2N KOH containing 150 mM TES and 0.3 M KCl. The samples were centrifuged at 20,000 g to remove precipitated potassium perchlorate. Extracts were flash-frozen in liquid nitrogen and lyophilized then reconstituted in 350 μL of a solution containing 25% D_2O and 5 mM EDTA for analysis by NMR.

NMR spectra were acquired on a Varian ANOVA 600 MHz superconducting magnet housed in the Max T. Rogers NMR Facility in the Department of Chemistry at Michigan State University. Fully relaxed spectra were the sum of 1024 data transients acquired with a $\pi/2$ pulse width and a pre-delay of 15 seconds at the phosphorus frequency (242.8 MHz) using a 48 KHz sweep width and 32 K points. Summed data were filtered with a 7.0 Hz exponential filter prior to the Fourier transform. Identification of spectral resonances was based on chemical shift differences against a

known standard (dilute H_3PO_4 placed in a separate concentric capillary centered in the NMR tube). Quantification was performed using the commercially available integration package.

Phosphorus NMR of INS-1 cell bioreactors

To measure the energetic changes associated with changes in glucose concentrations in vivo, INS-1 cells were washed once in NMR KRB and harvested mechanically by gently scraping the flask into 10 mL of low glucose NMR KRB. Cells were washed in NMR KRB, centrifuged at 600g for 4 min to a pellet then gently resuspended in 500 μL of low glucose NMR KRB. Meanwhile a loading system was assembled to assist in transferring the cell slurry to 1 mm ID membranous tubing (Spectrum Labs, Rancho Dominguez, CA) that is permeable to substrates and oxygen, thus creating a small cell bioreactor.

The loading system consisted of a 50 mm length of tubing, a 1 mL syringe (Becton Dickinson & Company, Franklin Lakes, NJ), a gel-loading pipette tip (Bio-Rad Laboratories, Hercules, CA), and a 20 mL container of low glucose NMR KRB. The permeable tubing was filled with NMR KRB and sealed at one end with heated forceps. A small hole was then created through the sealed end using a heated needle, and surgical thread was strung through the hole to aid positioning of the bioreactor within the probe. The sealed tubing was then fitted to the gel-loading tip, which had been attached to the 1 mL syringe. This apparatus was suspended vertically and the tubing lowered into the 20 mL of low glucose NMR KRB. Once the loading apparatus was set up, the cell slurry was transferred to the bottom of the 1 mL syringe using a long stem glass Pasteur pipette.

The syringe plunger was gently inserted to promote cell transfer from the syringe to the tubing. Pressure was constantly monitored until loading was terminated following the development of back pressure due to filling of the bioreactor. The bioreactors were trimmed to a fixed length (30 mm) and sealed with heated forceps.

Cell bioreactors were then loaded into a custom built NMR probe [88] consisting of a 7-turn solenoid of silver-coated copper foil tuned to the phosphorus frequency (162 MHz) by a balance matched tank circuit. Bioreactors were positioned inside a 1.5 mm x 1.8 mM (ID x OD) thin-walled capillary tubing and the bioreactor centered around the coil Once positioned, cells were superfused with NMR KRB equilibrated with 95% O_2/5% CO_2 at 30°C.

^{31}P-NMR spectroscopy experiments were performed on a 9.4 T widebore Bruker AM400 housed in the Molecular Imaging Research Center at Michigan State University. Prior to data acquisition, magnetic field homogeneity was optimized by shimming the available proton signal. Typical line widths for a loaded bioreactor were 60 Hz. ^{31}P scans of INS-1 cells consisted of 4096 data points collected using a sweep width of 4000 Hz with a $\pi/2$ pulse width (1.75 μs) and 3 second pre-delay. Data were summed to a total of 300 transients and processed by zero filling with 4096 points, apodization with a 25 Hz exponential filter then Fourier transformed.

Cells were initially superfused with low glucose NMR KRB for 96 min during serial NMR acquisition. A total of 6 NMR spectra were acquired under initial conditions before changing superfusate to high glucose NMR KRB. The superfusate was not recirculated and in some experiments fractions were collected to assay for insulin release. There was little difference in the rate of insulin release from INS-1 cells either superfused

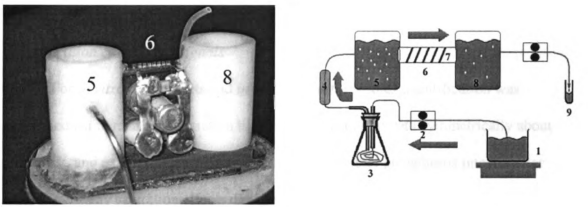

Figure 2: ^{31}P-NMR Probe Schematic
1) Kreb's Ringer Buffer 2) High Speed Pump 3) Oxygenator 4) 31° C water jacket 5) Pre-cellular oxygenated media 6) Eight turn silver-coated copper solenoid coil 7) Thin-walled capillary tube into which cells are placed 8) Post-cellular Media 9) Media collecting tube. Components 4-8 are contained within the NMR probe. Red arrows indicate direction of media flow.

in the NMR probe or incubated in cell culture (data not shown). The kinetics and steady-state levels of phosphorus containing metabolites were monitored during the entire time course. In some experiments the order of glucose challenge was reversed. Following the completion of each experiment, the portion of the bioreactor in the sensitive volume of the NMR coil was cut out and frozen in liquid nitrogen for subsequent PCA extraction, HPLC analysis of metabolites, and protein quantification.

Quantification of the NMR signals

For *in vitro* cell extracts and *in vivo* bioreactor studies quantification was performed using manual integration by summing the digital data symmetrically about each peak, and expressing each as a percentage of the total phosphorus integral after adjusting for any saturation effects due to the recycle delays used for these experiments (saturation factors: PPA=1.16+/-0.07, G6P=1.35+/-0.03, Pi=1.16+/-0.03, PCr=1.25+/-0.05, γATP=1.05+/-0.04, αATP=0.98+/-0.04, βATP=1.02+/-0.03). Metabolite values were calculated by normalizing each resonance to the mean of the γ,α and β ATP peaks then converted to chemical content by ratioing the remaining resonances to the mean ATP value determined by HPLC. In both the in vitro and in vivo studies the total phosphorus integral did not change over the time course of the experiments. Finally, this value was multiplied by the protein content of each bioreactor and divided by the cellular water content (0.80) [70].

Intracellular pH was calculated from the chemical shift differences between inorganic phosphate and PCr using the following equation:

$$pH = pK_a + \log\left[\frac{\delta_o - \delta_A}{\delta_B - \delta_o}\right]$$

where the temperature adjusted pK_a= 6.72, δ_A= 3.17, δ_B = 5.68 [89]. , and δ_o = observed chemical shift difference between Pi and PCr at 30 ± 1°C.

Free ADP and the ATP free energy were calculated using the following relations:

$$ADP_{free} = \frac{Cr * ATP}{PCr * Keq[H^+]} \quad \text{and} \quad \Delta G_{ATP} = \Delta G^{\circ}_{ATP} + RT * \ln\left[\frac{ADP * Pi}{ATP}\right]$$ where the K_{eq}

for creatine kinase (195) was adjusted for temperature according to Teague and Dobson [90] and ΔG°_{ATP}= -32.8 kJ/mol.

Metabolite Analysis

Metabolites were measured in duplicate by HPLC analysis of the neutralized perchloric acid extracts from both cultured cells and cell bioreactor NMR experiments. Chromatography was performed on a Shimadzu HPLC system consisting of an SCL-10A controller, two LC-10AD pumps, SIC-10AD AutoInjector, and SPD-10A/10AV UV-Vis Detector. Anion exchange HPLC was used to determine the concentrations of ATP and PCr while creatine was assayed by cation column HPLC using methods previously described with minor modifications [91]. Anion chromatography was performed on a Vydac Model 302IC4.6 and peaks were eluted using a linear phosphate gradient (KH_2PO_4) from 50 mM (pH=4.5) to 500 mM (pH=2.7) at a flow rate of 2.0 mL/min over 20 minutes and detected at both 210 (PCr) and 254 (ATP) nm and quantified against known standards. Cation exchange chromatography of creatine content was performed using a Phenomenex Phenosphere 5μm column with detection at 210 nm against known standards. The mobile phase was 25 mM NaH_2PO_4 pH=5.8 run isocratic. Protein levels for all experiments were determined by dissolving cellular protein in 1 mL of NaOH and utilizing the method of Lowry et al [92].

Insulin Measurements

For insulin secretion experiments, INS-1 cells were plated at $0.25x10^6$ cells per well in 12-well plates. On the 5^{th} day after plating, cells were changed to media containing 5 mM glucose with or without 40 mM creatine. On the 7^{th} day cells were rinsed twice with NMR KRB (no glucose), exposed to 2.0 mM glucose NMR KRB for 30 minutes, and challenged with 2.0 mM or 16.7 mM glucose NMR KRB for 1 hour. Media aliquots were diluted 1:20 in duplicate and insulin was quantified using a radioimmunoassay kit (RIA, LINCO Research, Inc., St. Charles, MO) (rat insulin) and normalized to cellular protein.

RESULTS

Insulin Secretion and Creatine Loading

Basal insulin production in INS-1 cells incubated in low glucose media was 97 +/- 23 ng/mg protein and increased over four-fold when incubated in high glucose media to 350 +/- 44 ng/mg protein (Figure 3). In contrast, when cells were first loaded with creatine (40 mM creatine media) there was a 50% decrease in the total release of insulin when incubated in low glucose to 44 +/- 11 ng/mg protein. There was also a correspondingly lower secretion observed for creatine-loaded INS-1 cells challenged with high glucose (162 +/- 19). Interestingly, while both the low and high glucose values for insulin secretion for creatine-loaded INS-1 cells were approximately half those for INS-1 cells that were not creatine treated, the relative increase in insulin secretion for the same glucose challenge was approximately four-fold in both cases (Figure 3 inset).

^{31}P-NMR of INS-1 cell extracts

Chemical changes in INS-1 cell extracts in response to high glucose challenge were investigated in both creatine-free and creatine-loaded cells grown in T-75 flasks using parallel experimental protocols to the insulin secretion studies. Neutralized perchloric acid extracts were generated from creatine-loaded INS-1 cells under high glucose challenge by acid quenching and examined by NMR spectroscopy using a recycle delay that was 5*T1 thus avoiding saturation effects (Figure 4). There was a large range in concentration of each metabolite illustrated by the broad range of signal

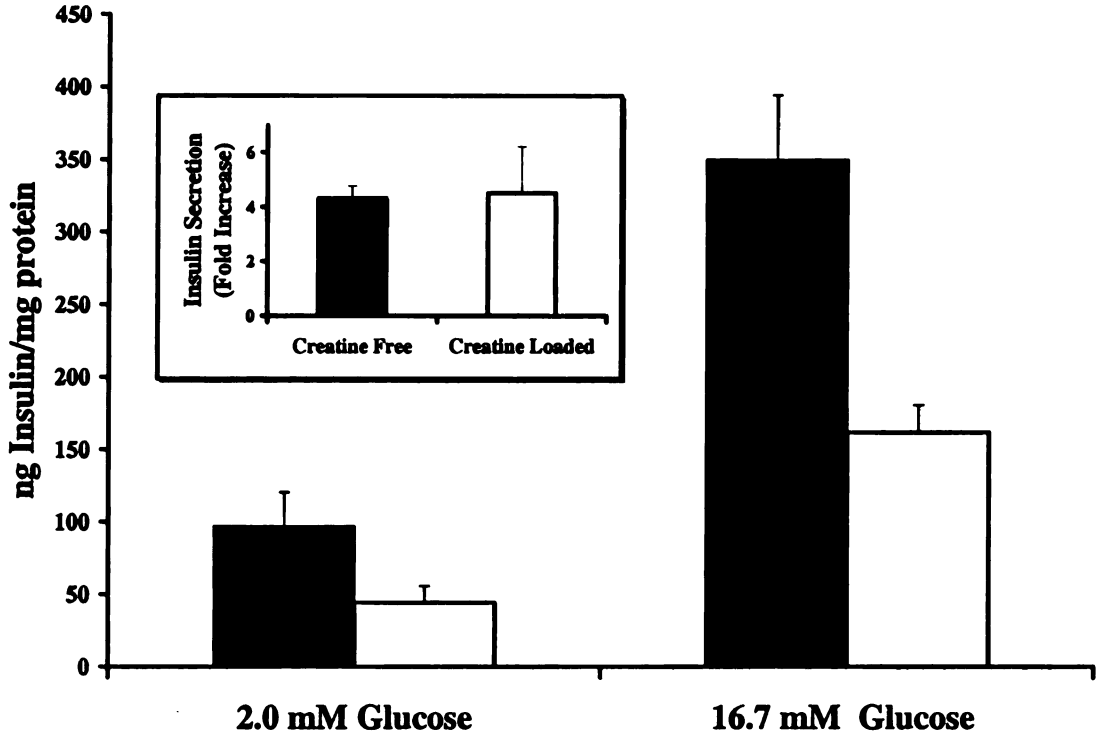

Figure 3: Insulin release from INS-1 cells

INS-1 cells (creatine free ■ , creatine-loaded □) were treated with 40 mM Creatine or left untreated for 48 hours before a 1 hour challenge with 2.0 mM or 16.7 mM glucose. The cell media was then analyzed by radioimmune assay for insulin levels and normalized to protein levels. Note that Cr loading decreased absolute insulin secretion under both basal and glucose stimulated conditions however the fold-increase appears to be unchanged (see inset). Results represent the average of 13-16 experiments +/- standard error of the mean (S.E.M.).

intensities from ADP and NAD(P)H to the glucose-6-phosphate. The inset presents the ratio of glucose-6-phosphate to phosphocreatine under these conditions. The steady-state phosphate potential resulting from the high glucose challenge is reflected in the metabolite changes measured in neutralized perchloric acid extracts of creatine-loaded INS-1 cells clamped at each glucose concentration (Figure 5). Changing the incubation medium from low to high glucose did not alter the cytosolic ATP content relative to the PPA external standard. In contrast, PCr and glucose-6-phosphate increased in response to elevated glucose, resulting in an increase in the calculated phosphate potential in cells treated with high glucose. The pattern of metabolite changes observed by NMR spectroscopy of extracts was qualitatively (but not quantitatively) similar in non-creatine loaded cells (data not shown) indicating these changes result from the increased glucose and not creatine content.

^{31}P-NMR of INS-1 cell bioreactors

To investigate the dynamics of energetic changes in INS-1 cells in response to a glucose challenge, serial ^{31}P-NMR measurements were performed. Figure 7 shows a stacked plot of serial NMR spectra of creatine-loaded INS-1 cells that were superfused with low and high glucose media followed by return to low glucose. As in cell extracts, following transition from low to high glucose, the cellular concentration of PCr increased. A similar increase occurred in glucose-6-phosphate but does not appear as dramatic in these experiments due to post-processing effects of exponential filtering on signal intensities. As in studies using cell extracts, there was no change in cytosolic ATP relative to the PPA external standard throughout the entire time course. In contrast to the

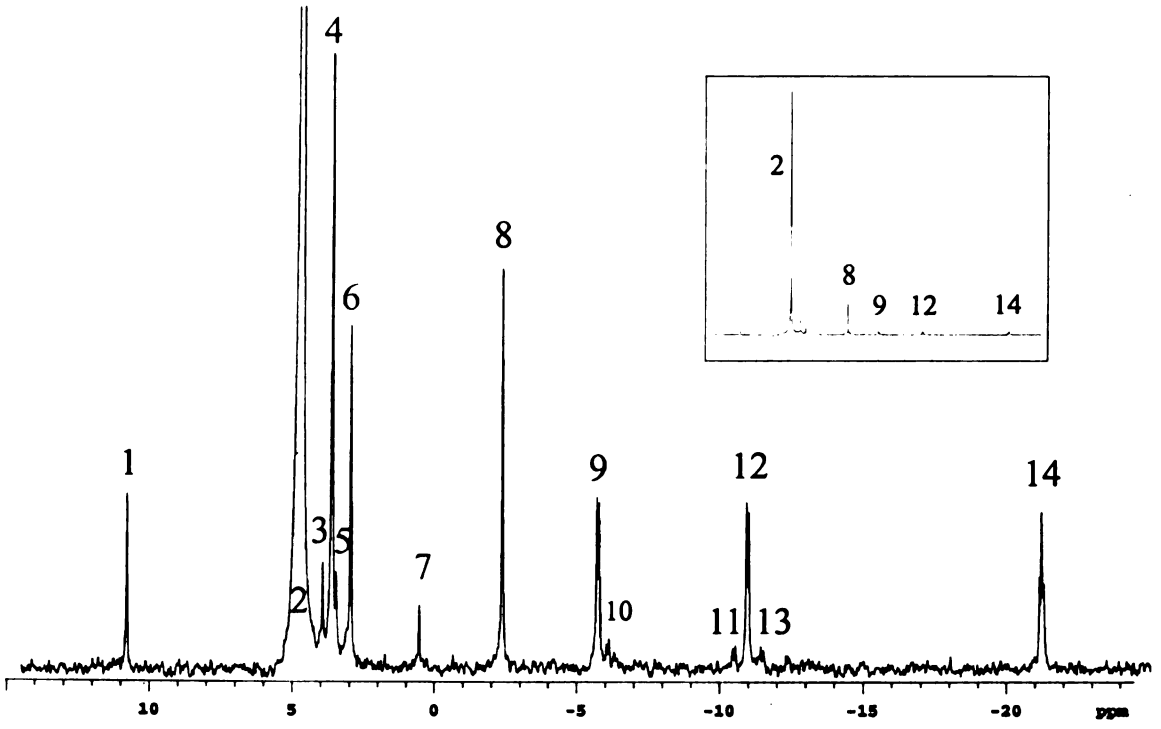

Figure 4: Phosphorus metabolite content in creatine loaded INS-1 cells from neutralized perchloric acid extracts.

^{31}P-NMR spectra of INS-1 cells loaded for 48 hours with 40 mM creatine and challenged with 16.7 mM glucose for 1.5 hours. Metabolite identification: 1=phenylphosphonic acid (10.95 ppm), 2=glucose-6-phosphate (5.27 ppm), 3=unidentified (3.98 ppm), 4=phosphocholine (3.75 ppm), 5=unidentified (3.42 ppm), 6=inorganic phosphate (3.11 ppm), 7=phosphodiester (0.685 ppm) 8=phosphocreatine (-2.52 ppm), 9=γATP doublet (-5.58, -5.66 ppm), 10=βADP doublet (-5.91, -5.99 ppm), 11=αATP doublet (-10.81, -10.88 ppm), 12=αADP doublet (-10.35, -10.43 ppm) 13=NAD(P)H (-11.30 ppm), 14 βATP triplet (-21.05, -21.13, -21.21 ppm). The inset shows the full size of the glucose-6-phosphate peak (2). The data was acquired using a Varian 600 MHz superconducting magnet with the following acquisition parameters: $\pi/2$ pulse width, 48573.2 Hz sweep, 32K digital points, 15s recycle time (5*T1), 1024 scans. Data were processed with a 7.0 Hz exponential filter prior to the Fourier transform.

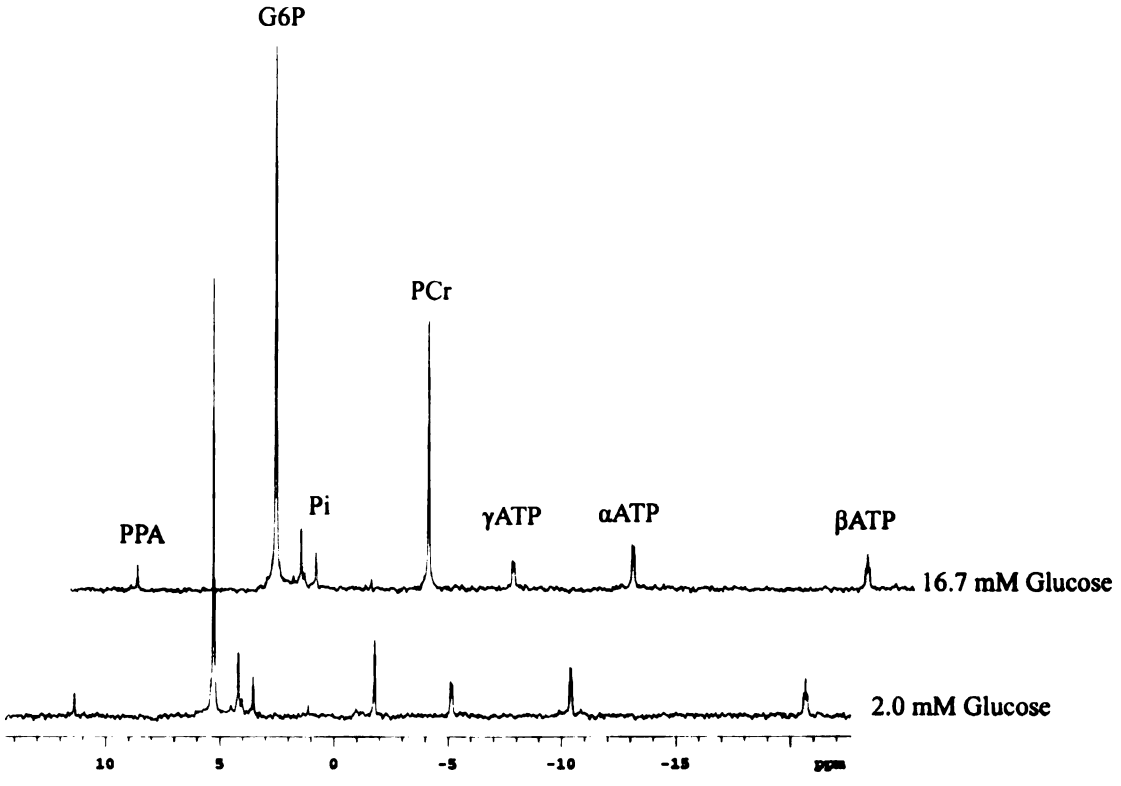

Figure 5: High Resolution NMR Spectra of INS-1 Cellular Extracts Treated in High and Low Glucose

INS-1 cells were creatine loaded for 48 hours and treated with 2.0 mM or 16.7 glucose 1.5 hours immediately before acid quenching. Comparison of the two spectra reveals a substantial increase in the PCr concentration without a significant change in the ATP concentration of cells exposed to high glucose compared to cells treated with low glucose. The spectra were gathered on a Varian 600 MHz superconducting magnet and represent the sum of 1024 scans spaced by a relaxation time of 0.5 seconds and the following acquisition parameters: $\pi/2$ pulse width, 48573.2 Hz sweep width, 32K digital points, 15s recycle time (5*T1). Data were processed with a 7.0 Hz exponential filter prior to the Fourier transform.

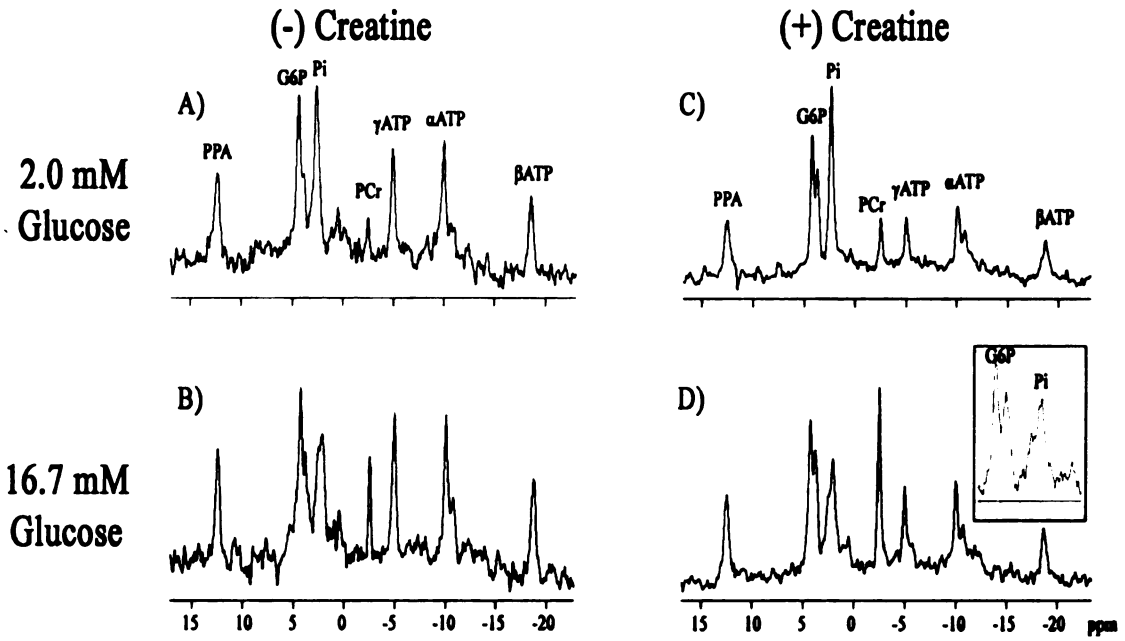

Figure 6: ^{31}P-NMR spectroscopy of INS-1 cell bioreactors
Both creatine loaded (C,D) and non-creatine loaded (A,B) INS-1 cells respond to a glucose challenge with reciprocal changes in Pi and PCr while ATP concentrations remain constant. Upon the transition from low to high glucose a decrease in Pi is associated with an increase in PCr without visible changes in ATP levels. The inset in D) demonstrates resolution of the G6P peak. Spectra are sums of 3 consecutive 16 minute experiments that each consist of 300 scans. Data was collected on a 9.4 T AM 400 MHz superconducting magnet with acquisition parameters: 4096 data points, 4000 Hz sweep width, $\pi/2$ pulse width (1.75 μs) and 3 second pre-delay. Data were summed to a total of 900 transients and processed by zero filling with 4096 points, apodization with a 25 Hz exponential filter before Fourier transformation.

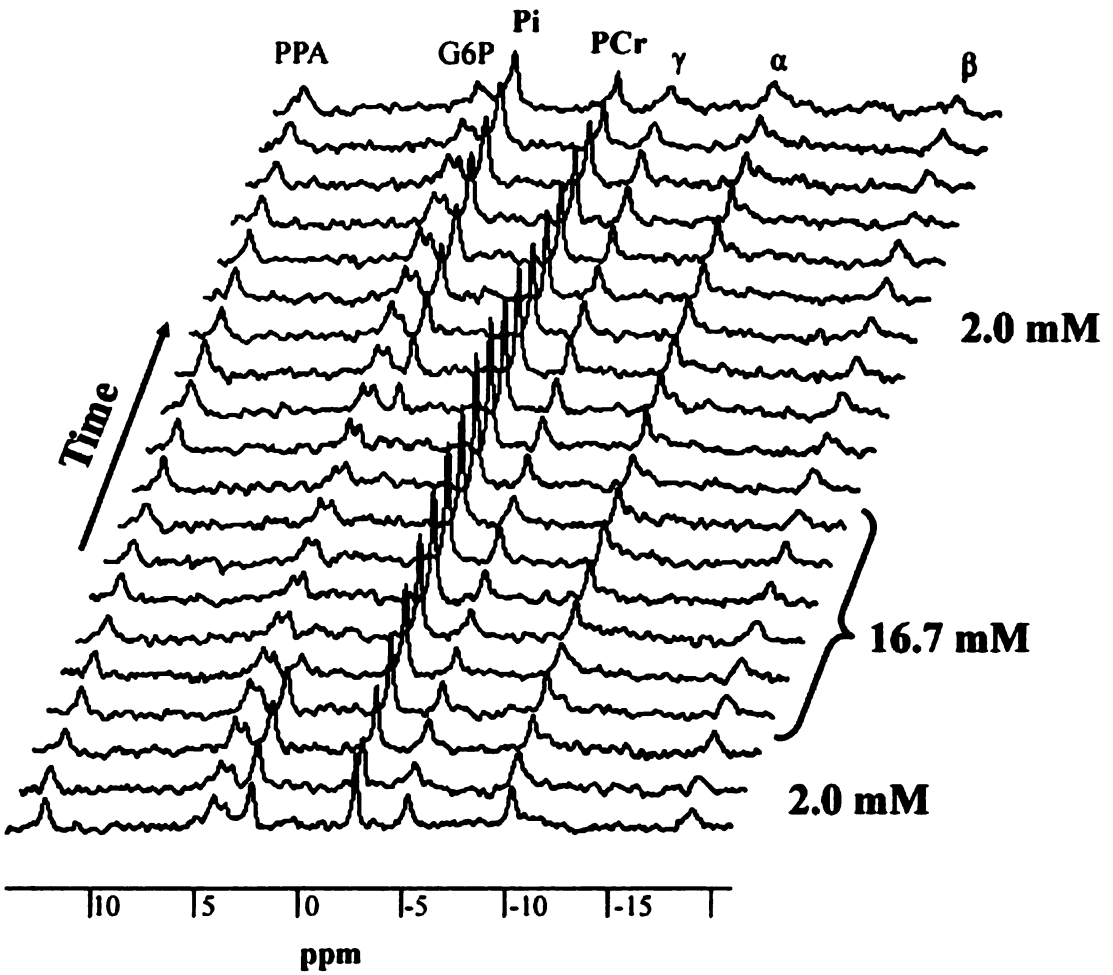

Figure 7: Serial ^{31}P-NMR spectra depicting the time course and reversibility of energetic changes occurring in INS-1 cells exposed to bracketing conditions of 2.0 mM - 16.7mM - 2.0 mM glucose

The energetic changes in INS-1 cells, as observed by changes in PCr and Pi, are reversible. PCr increases during the glucose challenge and eventually reverts to its original level following return to low glucose. Pi changes in the opposite direction, decreasing during the high glucose period and returning to higher levels when low glucose conditions are restored. ATP levels remain constant throughout the experiment. Each spectrum represents a 16 minute experiment consisting of 300 scans collected on a 9.4 T AM 400 MHz superconducting magnet with acquisition parameters: 4096 data points, 4000 Hz sweep width, $\pi/2$ pulse width (1.75 μs) and 3 second pre-delay.

cell extract spectra, Pi decreased following a glucose challenge. This change may not have been visible in cell extracts due to hydrolysis during cell harvesting. These changes were fully reversible when returned to low glucose superfusate and returned to the same value but with a slower time course. The total phosphorus integral did not change in these experiments (Tables I, II, and III).

Analysis of the kinetic changes in metabolites are presented in figure 8 expressed as the PCr/Pi ratio. Steady-state superfusion with low glucose resulted in a sustained PCr/Pi ratio of 1.0. Transition from low to high glucose resulted in an increase in the PCr/Pi ratio to 3.5 which was complete within 45 min. This PCr/Pi ratio was sustained at 3.5 as long as superfusion with high glucose continued. Returning the INS-1 cells to low glucose resulted in concomitant decreases in the PCr/Pi ratio to 1.0 but with a time course that occurred over 192 min. Quantitative analysis of the metabolite contents in INS-1 cell bioreactors were performed at the sustained steady-states for each glucose concentration in both creatine-free and creatine-loaded cells by summing serial spectra at each glucose concentration where the metabolite levels remained stable. Figure 6 shows representative NMR spectra from creatine-free (-) and creatine-loaded (+) INS-1 cells under both low and high glucose challenge (summed data from 48 min).

Taken together with the HPLC analysis of the perchloric acid extracts of each cell population from the NMR experiment, the in vivo phosphorus metabolite contents (ATP, ADP, PCr and free Cr) as well as intracellular pH and the calculated phosphate potentials are presented in Table I (creatine-free), Table II (creatine loaded), and Table III (creatine loaded/reverse glucose challenge) . Creatine loaded INS-1 cells did increase overall metabolite content irrespective of the superfusate glucose concentration. Total creatine

Table I: Metabolic Changes in Creatine-Free INS-1 Cells Following Transition From Low to High Glucose

	2.0 mM Glucose			16.7 mM Glucose			Δ
Pi	**2.17**	+/-	0.32	**1.67**	+/-	0.27	**-0.50**
PCr	**0.52**	+/-	0.07	**0.70**	+/-	0.13	**0.17**
ATP	**1.51**	+/-	0.13	**1.51**	+/-	0.15	**0.00**
Cr	**3.32**	+/-	0.28	**2.75**	+/-	0.23	**-0.56**
Total	**10.31**	+/-	1.25	**9.70**	+/-	1.22	**-0.61**
ADP (uM)	**47.86**	+/-	6.99	**32.57**	+/-	1.75	**-15.29**
ΔG_{ATP} (kJ/mol)	**-57.25**	+/-	0.55	**-58.80**	+/-	0.14	**-1.56**
pH_i	**7.21**	+/-	0.03	**6.93**	+/-	0.03	**-0.28**

Table II: Metabolic Changes in Creatine-Loaded INS-1 Cells Following Transition From Low to High Glucose

	2.0 mM Glucose			16.7 mM Glucose			Δ
Pi	**4.02**	+/-	0.92	**2.50**	+/-	0.73	**-1.52**
PCr	**1.78**	+/-	0.21	**3.71**	+/-	0.37	**1.94**
ATP	**1.73**	+/-	0.13	**1.69**	+/-	0.12	**-0.03**
Cr	**22.16**	+/-	1.69	**17.43**	+/-	1.33	**-4.73**
Total	**15.77**	+/-	1.50	**15.76**	+/-	1.41	**-0.01**
ADP (uM)	**118.66**	+/-	17.66	**44.51**	+/-	8.07	**-74.15**
ΔG_{ATP} (kJ/mol)	**-53.88**	+/-	0.68	**-57.85**	+/-	1.08	**-3.97**
pH_i	**7.08**	+/-	0.01	**6.81**	+/-	0.02	**-0.27**

Table III: Metabolic Changes in Creatine-Loaded INS-1 Cells Following Transition From High To Low Glucose

	16.7 mM Glucose			2.0 mM Glucose			Δ
Pi	**5.85**	+/-	1.37	**10.18**	+/-	2.26	**4.33**
PCr	**6.75**	+/-	1.64	**2.49**	+/-	0.40	**-4.26**
ATP	**1.74**	+/-	0.17	**1.70**	+/-	0.16	**-0.03**
Cr	**17.51**	+/-	1.73	**22.26**	+/-	2.20	**4.75**
Total	**22.13**	+/-	4.12	**22.16**	+/-	3.70	**0.03**
ADP (uM)	**25.33**	+/-	1.83	**83.32**	+/-	6.35	**57.99**
ΔG_{ATP} (kJ/mol)	**-56.77**	+/-	0.36	**-52.31**	+/-	0.49	**4.46**
pH_i	**6.75**	+/-	0.01	**6.97**	+/-	0.05	**0.22**

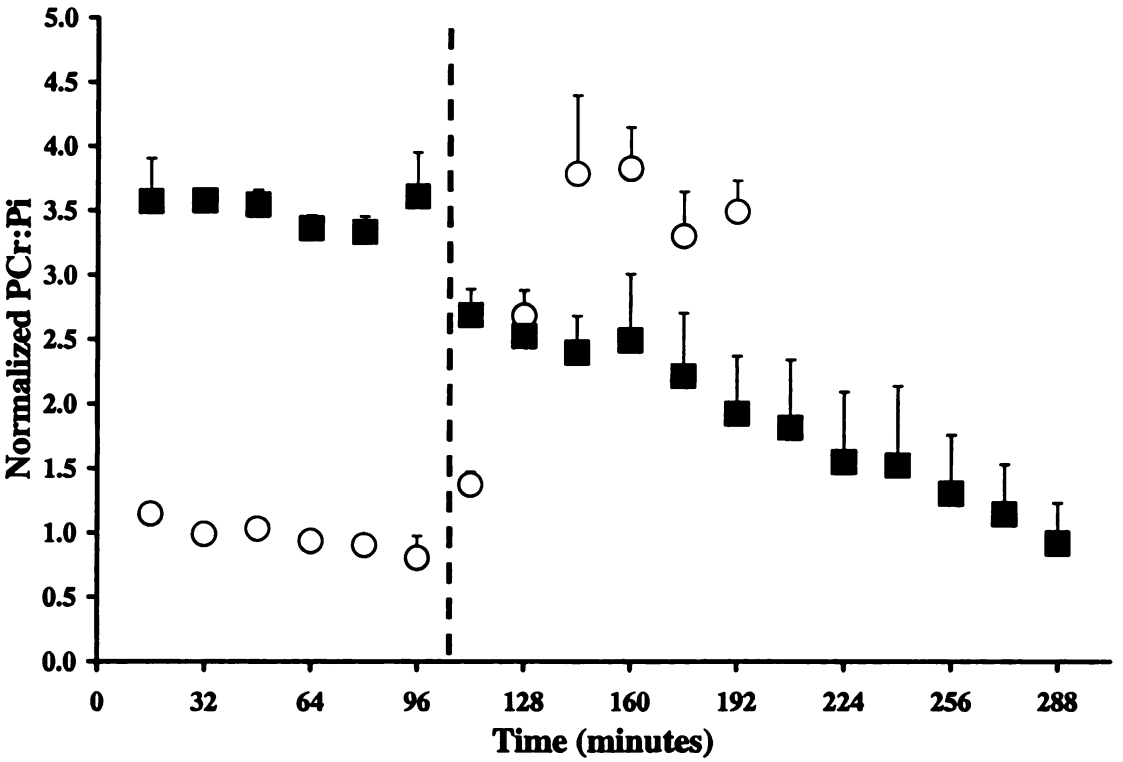

Figure 8: Time course and reversibility of energetic changes occurring in INS-1 cells before, during, and after a glucose challenge as reflected by change in the PCr:Pi ratio

Creatine loaded INS-1 cells transitioned from low (2.0 mM) to high (16.7 mM) glucose (○) exhibit an increase in the PCr:Pi ratio from a normalized steady state ratio of 1.0 to a new steady state ratio of approximately 3.5 that takes 45 minutes to complete as determined by serial ^{31}P NMR spectra. Cells initially treated with high glucose (■) begin with a steady state ratio of PCr:Pi approximately equal to the ending ratio for the reciprocally treated cells (3.5). When media glucose is lowered, these cells exhibit a prolonged decrease in the PCr:Pi ratio back to 1.0 that occurs over 192 minutes. Results are the average of 3-4 experiments +/- S.E.M.

content was increased by 6-fold with creatine loading but the relative distribution between free and phosphorylated creatine was dependent on glucose treatment. Increasing glucose from low to high treatment in creatine loaded cells resulted in a two-fold increase in PCr and a similar decrease in creatine. These effects were fully reversible. Intracellular pH decreased from 7.21 +/- 0.03 to 6.93 +/- 0.03 in response to increased glucose concentration and was relatively unaffected by creatine-loading. The change in pH_i was not significantly different in both creatine-free and creatine-loaded INS-1 cell populations and was fully reversible on return to low-glucose medium (Tables I, II, and III). Free ADP concentrations were calculated from the metabolite contents measured by NMR and HPLC and using the creatine kinase equilibrium constant adjusted for temperature. Free ADP was higher in 2 mM glucose in both creatine-free and creatine-loaded INS-1 cells (47.86 and 118.66 μM respectively). Switching to high glucose superfusate resulted in decreasing free ADP to 32.57 +/- 1.75 and 44.51 +/- 8.07 μM for creatine-free and creatine-loaded cells respectively. The changes in free ADP together with decreasing Pi content resulted in calculated free energy changes of -1.56 and -3.97 kJ/mol for creatine-free and creatine-loaded INS-1 cells respectively (Tables I and II). All metabolic changes were fully reversible following the removal of a glucose challenge (Table III).

Insulin release and phosphorus metabolite changes

In response to increased glucose, ATP remained unchanged relative to the PPA resonance. However ADP decreased with elevated glucose concentration as insulin release increased. The relationship between free ADP and insulin release in creatine-free

and creatine-loaded INS-1 cells is presented in Figure 9. Correlations of the decreased free ADP with an increase in PCr are coupled through creatine kinase equilibration. However, as noted in the data in Tables I and II, the increases in PCr occurred with concomitant changes and stoichiometric decreases in the Pi resonance. Because Pi is also altered by the glucose challenges imposed in creatine-free and creatine-loaded INS-1 cells a more comprehensive description of the metabolite changes associated with insulin release takes the form of the ATP free energy (or phosphate chemical) potential (ΔG_{ATP}). The relationship between ΔG_{ATP} and insulin released is plotted in the inset of Figure 9.

Time Course of Glucose Induced Metabolic Changes

Figure 10 explores the relationship between three indicators of the cellular energetic status (PCr:Pi ratio, free ADP, and intracellular pH). In response to a glucose challenge PCr increased and Pi decreased, resulting in an increase in the PCr:Pi ratio from 1.0 to 3.5 over the course of 48 minutes. These '^{31}P-NMR observable' changes were used to calculate a depression in free ADP from 118 to 44 μM that reached a new minimum in approximately the same timeframe. Finally, a decrease in intracellular pH from 7.1 to a new steady state of 6.8 was calculated from NMR spectra (see methods). Again, this reversible glucose induced change occurred during a comparable time course to other metabolic changes.

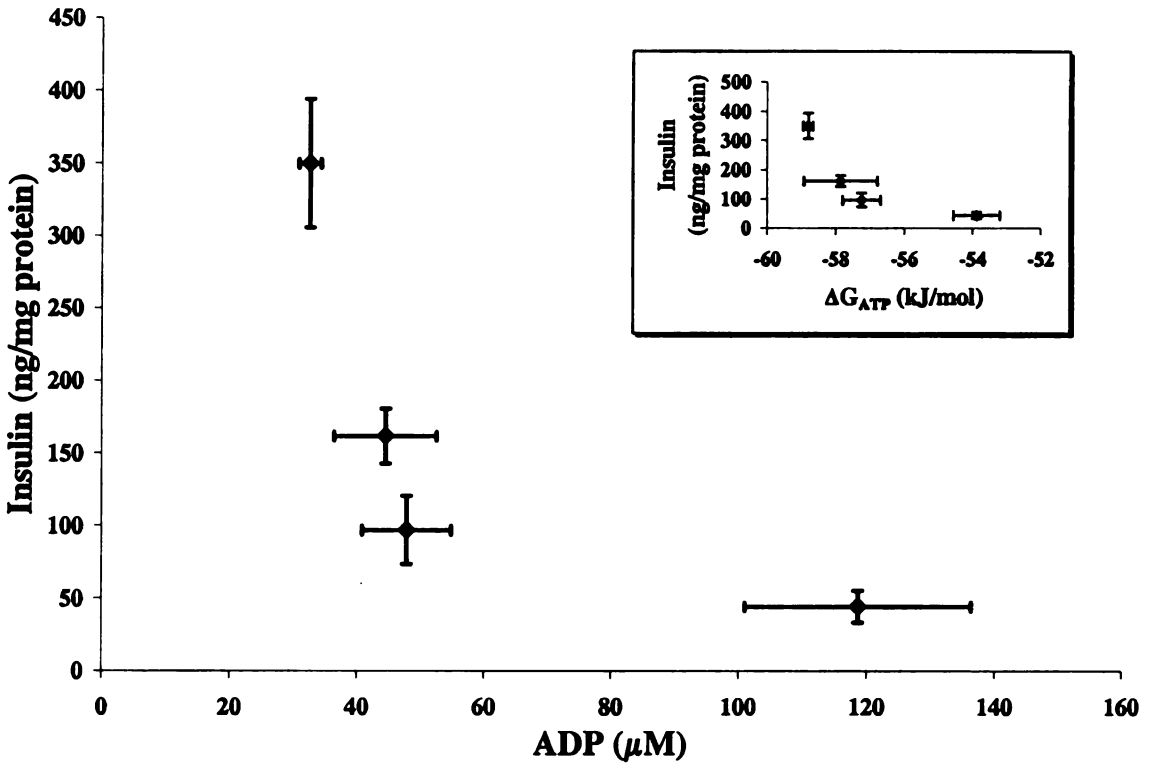

Figure 9: Relationship between steady state free ADP and glucose induced insulin secretion

The concentration of free ADP, as mediated by glucose stimulation and creatine loading, is negatively correlated with insulin secretion. A glucose challenge promotes a decrease in free ADP, which is associated with an increase in insulin secretion. Free ADP was calculated from the creatine kinase equilibrium using the average [PCr], [ATP], [Pi] measured from 48 minutes of steady state NMR spectra of four experiments and [Cr] determined by HPLC. Insulin secretion was calculated from parallel experiments of plated INS-1 cells that were also creatine loaded and exposed to identical challenging concentrations of glucose. As demonstrated in the inset, a similar relationship exists between insulin secretion and the free energy of ATP (ΔG_{ATP}), a more comprehensive measurement of cellular energetics that incorporates [Pi]. Insulin data is the result of 13-16 experiments +/- S.E.M. while ADP values were averaged from 4-5 experiments +/- S.E.M.

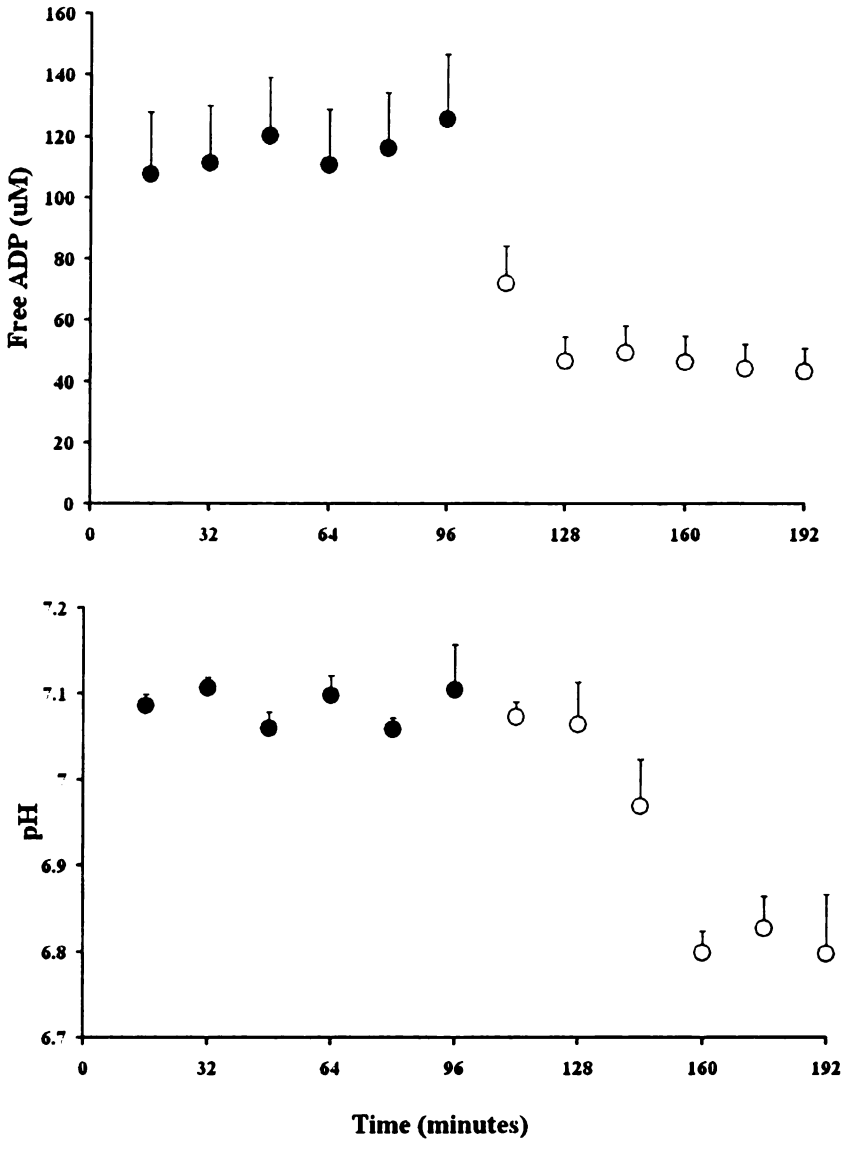

Figure 10: Kinetic and quantitative relationship between free ADP and intracellular pH following a glucose challenge

Creatine loaded INS-1 cells were superfused with 2.0 mM glucose NMR KRB for 96 minutes (●), followed by a 96 minute glucose challenge with 16.7 mM glucose NMR KRB (○). After the transition to high glucose free ADP decreased. Concomitantly the intracellular pH decreased. Free ADP reaches a steady state approximately 30-45 minutes after exposure to high glucose while peak intracellular acidification occurs after 1 hour. Results are the average of five experiments +/- S.E.M.

DISCUSSION

The purpose of this study was to characterize the energetic changes associated with glucose induced insulin secretion. The two main findings are 1) glucose induced insulin secretion is energetically associated with a decrease in ADP and 2) the energetic changes associated with insulin secretion reach a new steady state maximum 48 minutes after a sustained glucose challenge. The implications of these findings for β-cell function and type II diabetes are discussed below along with proposals for future experiments.

Adenine Nucleotide Changes: Importance of ADP

In contrast to common understanding of glucose induced insulin secretion, we did not observe an increase in ATP following a glucose challenge (Figure 7, Tables I-III). Instead we calculated a decrease in free ADP to a new steady state concentration subsequent to glucose stimulation (Tables I-III). Further, an inverse correlation between steady state ADP and insulin secretion was demonstrated in creatine and non-creatine loaded cells (Figure 9), suggesting that ADP may be the critical determinant of K^+_{ATP} channel gating and subsequent insulin secretion. High basal levels of ADP stabilize the open state of the K^+_{ATP} channel. Following a glucose challenge, mitochondrial metabolism induces a decrease in ADP, mediated by the creatine kinase equilibrium, thus promoting closure of the K^+_{ATP} channel, increasing $[Ca^{2+}]_i$, and inducing insulin secretion. This model is consistent with previously discussed experiments demonstrating inhibition of glucose induced insulin secretion by mitochondrial inhibitors [22] [23] [39], all of which would increase free ADP. Manipulation of adenine nucleotide levels apart

from mitochondrial inhibition has been difficult to achieve, but in our experiments, Cr loading appeared to increase ADP levels and thereby depress insulin secretion.

Creatine loading decreased basal and glucose induced insulin secretion. Based on our data, we suggest that this creatine effect on insulin release may be due to the apparent creatine induced increase in free ADP. The mechanism whereby creatine loading increases basal ADP is unclear. Complete validation of this mechanism could come from experiments determining insulin secretion and free ADP levels from islets exposed to a range of submaximal glucose concentrations. Re-evaluation of such experiments from the literature found that a similar relationship between ADP and insulin secretion over a range of glucose concentrations was demonstrated, but not explicitly recognized, in mouse islets [64] and βHC9 cells [70]. A range of Cr concentrations could also be used to determine if a dose dependent relationship exists between Cr loading and [ADP]/insulin secretion.

Decreases in free ADP following a glucose challenge were calculated based on several ^{31}P-NMR observable energetic changes. Transition from low to high glucose induced an increase in PCr associated with a decrease in Pi and Cr (Figure 9, Tables I-III). Further, metabolite values returned to their normal levels following the restoration of basal glucose levels demonstrating that the observed energetic alterations were not simply artifacts of the bioreactor cellular environment. Our data is supported by similar glucose induced increases in PCr in extracts of rat pancreatic islets [85] and reciprocal changes in PCr and Pi in creatine loaded βHC9 cells [66].

The possibility that our system was inadequately sensitive to measure actual changes in ATP cannot be categorically excluded, but the obvious changes in PCr, Pi,

and Cr argue against this proposal. Another alternative is that the glucose challenge increased the frequency of ATP oscillations (occurring in seconds); evidence of this increase would have been obscured by the time resolution of our spectra (16 minutes). Another caveat in measuring intracellular ATP is that it is constantly being hydrolyzed to ADP for cellular energy. Therefore a time averaged measurement reflects the balance between the production and consumption of ATP. Glucose induced insulin secretion undoubtedly involves an increase in ATP production as well as an increase in ATP consumption (Na^+/K^+ ATPase, SERCA, etc). Based on our results, it appears that consumption of ATP by ATPases and creatine kinase is roughly equal to ATP production following a glucose challenge, thus the glucose induced average ATP is relatively constant, leaving ADP as the critical regulator of K^+_{ATP} channel gating and subsequent insulin secretion. Implicit in this conclusion is the importance of mitochondrial glucose metabolism, leading to the prediction that compromise of mitochondrial function could be involved in hyposecretion of insulin and type II diabetes. Loss of ATPase activity could also be involved in uncoupling of glucose induced insulin secretion. Indeed, ouabain, an inhibitor of the Na^+/K^+ ATPase, induces K^+_{ATP} channel closure and insulin secretion by preventing ATP hydrolysis and maintaining ADP at a low level [93] [94]. Activation of ATPases by a glucose challenge could also act as an 'off-switch' for insulin secretion. During a brief glucose challenge ATP production and consumption would increase to produce a decrease in ADP and induce insulin secretion. When the glucose challenge is removed, ATP production may decline before ATPase activity, driving an increase in ADP to reopen K^+_{ATP} channels, restrict triggering $[Ca2+]_i$, and terminate

insulin secretion. The time course of ADP changes may also explain the biphasic nature of glucose induced insulin secretion.

Time Course of Energetic Changes: 1st and 2nd Phase Insulin Secretion

Glucose induced energetic changes are immediately initiated following the transition from low to high glucose and reach steady state peak levels after a period of approximately 48 minutes (Figure 10). The energetic kinetics may be important for understanding 1st and 2nd phase insulin secretion in rat islets. 1st phase insulin secretion peaks approximately five minutes after a glucose challenge and is critically dependent on elevated $[Ca^{2+}]_i$. Artificial elevation of $[Ca^{2+}]_i$ in the absence of glucose metabolism can induce this 1st phase of insulin secretion. Physiologically, it is generally agreed that glucose metabolism elevates $[Ca^{2+}]_i$ by increasing the ATP:ADP ratio, thereby closing the membrane K^+_{ATP} channel, depolarizing the beta cell and opening voltage gated Ca^{2+} channels. Although the mechanism is unclear, it appears that $[Ca^{2+}]_i$ triggers the exocytosis of a subset of insulin granules that are docked and primed at the cell membrane (IRP). Although our time resolution is not optimal, a decrease in ADP was observed within 16 minutes of a glucose challenge. Assuming this ADP decrease is actually initiated immediately following the fuel stimulus, it could account for K^+_{ATP} channel closure and the triggering $[Ca^{2+}]_i$ for 1st phase insulin secretion. Alternatively, time averaging in the initial 16 minute spectrum could mask a vigorous but brief glucose induced increase in ATP:ADP or an unidentified "^{31}P NMR invisible" signal could be the causative agent in elevating $[Ca^{2+}]_i$ and inducing 1st phase insulin secretion. Based on our data, we conclude that ADP is the probable regulator of membrane K^+_{ATP} channel closing and 1st phase insulin secretion. This proposed relationship between ADP and β-

cell electrical activity does not rule out a role for additional signals that can amplify the underlying ADP energetic regulation of insulin secretion.

Despite the fact that $[Ca^{2+}]_i$ remains elevated in the beta cell for the duration of a glucose challenge, insulin secretion begins to decline following the initial peak at 5 minutes. This decline is ascribed to the depletion of the IRP. After reaching a nadir, insulin secretion begins to increase again reaching a sustained peak around 45 minutes after a high glucose infusion. This 2nd phase of insulin secretion is dependent upon signals generated by glucose metabolisms that are proposed to replenish the IRP via docking and priming of the reserve pool and/or RRP. In this model of 2nd phase insulin release, docking and priming represent rate limiting steps for $[Ca^{2+}]_i$ sensitive exocytosis. Among possible signals generated by glucose metabolism, mitochondrially regulated adenine nucleotides are proposed candidates for replenishing the IRP and promoting 2nd phase insulin release. In support of this proposition is data demonstrating the attainment of a new steady state peak in mitochondrial respiration (determinant of ATP and ADP levels) 45 minutes after exposure of rat islets to high glucose [21].

Our data clearly demonstrates a decrease in ADP that reaches a maximum approximately 48 minutes after a glucose challenge in INS-1 cells, concomitant with the attainment of peak 2nd phase insulin secretion in rat islets (Figure 10). Recent evidence demonstrating the localization of K^+_{ATP} channels to insulin granule membranes provides a possible mechanism for the metabolic regulation of 2nd phase insulin secretion.

K^+_{ATP} channels, composed of the SUR1A and $K_{IR}6.2$ subunits, are sensitive to pharmacological treatment with a group of drugs collectively referred to as sulfonylureas. Sulfonylureas promote insulin secretion by inducing K^+_{ATP} channel closure through

binding of the SUR1A subunit [95]. They are therefore commonly used to treat type II diabetes. Due to the relative inability of most members of this family of drugs to diffuse through the plasma membrane, the insulin secretory actions of sulfonylureas have traditionally been attributed to binding of plasma membrane SUR1A subunits [96]. Early evidence that the plasma membrane may not be the only, or even primary, site for K^+_{ATP} channel localization came from studies with radiolabeled sulfonylureas. Specifically, glibenclamide, one of the most potent secretion inducing sulfonylureas [97], was found to readily diffuse across the cell membrane [98]. When applied extracellularly >90% of the labeled glibenclamide mapped to intracellular structures, especially insulin granules [99]. This suggested that part of glibenclamide's therapeutic efficacy was due to its ability to activate insulin secretion via an intracellular (possibly K^+_{ATP} dependent) mechanism, thereby affecting the secretory competency of insulin granules. Indeed, Geng et al recently demonstrated that the majority of K^+_{ATP} channels expressed by β-cells are actually localized to the surface of insulin granules [100].

A role for granular K^+_{ATP} channels in vesicle trafficking and glucose induced insulin secretion has been hypothesized based on data obtained from experiments using the whole cell configuration that all permits intracellular application of a sulfonylurea and manipulation of metabolite levels. Intracellular infusion of tolbutamide, a common sulfonylurea, increases exocytosis as measured by changes in membrane capacitance [101]. The potentiation of insulin secretion by tolbutamide is dependent on the presence of triggering $[Ca^{2+}]_i$, binding of tolbutamide to intracellular K^+_{ATP} channels, induction of a granular Cl^- current, and insulin granule acidification. Use of Ca^{2+} free media or intracellular treatment with diazoxide, a K^+_{ATP} channel opener, prevents stimulation of

exocytosis by tolbutamide. Similarly, infusion of a Cl^- channel blocker (4,4'-dilsothiocyanostilbene-2,2'-disulfonic acid) or an antibody against the ClC-3 chloride channel blocked the ability of tolbutamide to promote insulin secretion [12]. Finally, tolbutamide was demonstrated to accelerate intragranular acidification, and any treatment that prevented acidification also prevented tolbutamide induced insulin secretion [12]. From this data it was proposed that a K^+_{ATP} associated chloride channel provides a countercurrent that promotes granule acidification through the V-type H^+-ATPase channel. $[Cl^-]_i$ would lower the electrochemical barrier for H^+ entry, thereby decreasing the pH of insulin granules and promoting 2nd phase insulin secretion via granular priming.

The mechanism whereby acidification promotes granular priming is unclear, but it has been proposed that a low pH could induce conformational changes in SNARE proteins that are favorable for granule priming and exocytosis. Under this proposed mechanism, opening of the chloride channel by a decrease in ADP would represent the rate-limiting step for 2nd phase insulin release and allow one energetic signal (free ADP) to regulate 1st and 2nd phase insulin secretion. Indeed, intracellular application of 5 mM Mg-ADP lowered Ca^{2+}/ATP induced insulin secretion and associated granule acidification [4]. Elevated $[Mg\text{-}ADP]_i$ did allow a small increase in Ca^{2+}/glucose induced exocytosis that may correspond to release of the previously primed granules responsible for 1st phase insulin secretion. Our data is completely consistent with this proposal, demonstrating a steady decrease in $[ADP]_i$ that reaches a minimum after 48 minutes of a glucose challenge (Figure 10). The steady state minimum level of free [ADP] would therefore set the rate of sustained 2nd phase insulin secretion by establishing the maximal rate of granule acidification and priming.

This mechanism could also explain the insulin secretory phenomenon known as time dependent potentiation (TDP). TDP of glucose induced insulin secretion occurs when previous exposure of β-cells to glucose increases insulin secretion to a subsequent stimulatory glucose dose [102]. The initial glucose exposure could increase the RRP and IRP of granules by promoting granule priming via decreased ADP and intragranular acidification. The prolonged kinetics of the PCr:Pi decrease following removal of stimulatory glucose could also be significant for TDP. The energetic signals of a previous glucose challenge may not be completely dissipated before the next glucose challenge due to the extended 'off rate' (Figure 8). The subsequent glucose challenge would, in addition to releasing a larger pool of immediately releasable granules, generate energetic/stimulatory signals from a higher baseline.

A potential hindrance to fully supporting this 2nd phase priming mechanism based on our data is that INS-1 cells have a relatively weak 2nd phase of insulin secretion compared to rat islets [2]. However, the lack of vigorous 2nd phase insulin secretion in INS-1 cells cannot rule out the possibility that the important signals (time-dependent decrease in ADP) are still appropriately generated, but the downstream mechanisms expressed in native β-cells are not present in the insulinoma cells. ^{31}P-NMR analysis of bioreactors containing rat islets would be needed to further explore this hypothesis. If a prolonged decrease in ADP was physiologically important for 2nd phase insulin secretion one would expect to observe a decrease in ADP peaking at 45 minutes concomitantly with peak respiration and peak 2nd phase insulin secretion. The heterogeneity of cell types in isolated islets could interfere with these measurements.

The role of granular K^+_{ATP} channels in priming and 2nd phase (amplifying) insulin secretion was recently questioned by characterization of SUR1 knockout mouse islets functionally lacking K^+_{ATP} channels [103]. In the experiments performed by Nenquin et al, islets isolated from SUR1 knockout mice demonstrated an increased basal level of insulin secretion, consistent with an inability to regulate cell depolarization. Surprisingly, the mutant β-cells responded to a glucose challenge by raising $[Ca^{2+}]_i$ and secreting a significant amount of insulin sustained over the duration of glucose infusion. Thus it would seem that granular K^+_{ATP} channels are not important for the amplifying/2nd phase of insulin secretion. These results, however, cannot exclude a role for ADP in regulating insulin granule priming and 2nd phase insulin secretion. One possibility is that a different sulfonylurea isoform is present in insulin granule membranes whose expression is unaffected by SUR1 knockout. Additionally, there may be another K^+_{ATP} independent sensor of ADP on granular membranes or the mutant β-cells may compensate for the loss of SUR1 in a currently uncharacterized manner.

Intracellular pH and 2nd Phase Insulin Secretion

Although the concentration of Pi in insulin granules was too low to measure by ^{31}P-NMR, thereby preventing real-time measurement of granular pH, we were able to measure the overall intracellular pH before and after a glucose challenge. Previous reports concerning the effects of glucose metabolism on intracellular pH in islets and model β-cells have been mixed [104-106]. Interpretation of these results is confusing at best due to the use of permeable pH sensitive dyes, permeable buffers, and variable media formulas. Our data, however, demonstrates unequivocally that sustained

acidification (~0.28 pH units) of the INS-1 intracellular environment is induced by a glucose challenge (Table I). This acidification is fully reversible and follows the time course of phosphate potential changes, reaching a new steady state low slightly after the nadir in free [ADP] (Figure 10). This cytoplasmic decrease in pH could indirectly contribute to granule priming and 2nd phase insulin secretion by influencing the granular pH. Indeed it has been clearly demonstrated that glucose induced insulin secretion is optimized by mild intracellular acidification (~0.2-0.5 pH units) [107, 108] and TDP is critically dependent on lowered intracellular pH [109]. The mechanism of this low pH_i induced potentiation is unclear but may be due to increasing the IRP of granule through pH sensitive priming. Several additional mechanisms have been proposed. An obvious candidate, the K^+_{ATP} channel, has been shown to be pH sensitive, but protons increase the activity of the channel, thereby opposing insulin secretion [110]. Activation of metabolic enzymes by low pH has also been suggested. Indeed, several mitochondrial enzymes (α-ketoglutarate dehydrogenase and isocitrate dehydrogenase) are upregulated by low pH, but the overall effect of this control on insulin secretion is unclear [111]. Although the relationship between intracellular acidification, depression of free [ADP], and priming induced 2nd phase insulin secretion cannot be explicitly established based on this data, the associated time course of each change supports this as a plausible exocytotic mechanism that remains to be tested. Inadequate control or generation of these energetic signals (especially ADP) regulating insulin secretion could therefore have implications for the development of type II diabetes.

Implications for Diabetes

The time course of metabolic defects responsible for inadequate glucose uptake in type II diabetes has long been debated from two different perspectives: 1) peripheral insulin resistance is the initial defect that leads to exhaustion of the β-cell secretory capacity or 2) the β-cell's ability to secrete insulin is initially affected leading to hypoglycemia and insulin resistance. Currently there is good evidence to suggest that both 1st and 2nd phase insulin secretion are compromised in pre-diabetics long before development of insulin resistance and overt diabetes [112, 113]. The cause of this secretory deficiency is unknown but our data suggests that it may be broadly related to a compromise in mitochondrial regulation of free ADP. Indeed, a large body of evidence supports the hypothesis that mitochondrial function in β-cells is correlated with the development and symptomatic presentation of type II diabetes. Specifically 1) A rare form of diabetes due to inadequate insulin secretion is caused by mutations in maternally inherited mitochondrial DNA [114]. 2) Mitochondria from diabetic patients have structural abnormalities and impaired oxidative capacity [115]. Oxidative phosphorylation genes are downregulated [116] and the activities of ATP synthase and creatine kinase are decreased in diabetic skeletal muscle [117]. Similar abnormalities are observed in obese patients. 3) Peterson et al [118] demonstrated that young, lean, insulin-resistant offspring of type II diabetics have a 30% slower rate of mitochondrial ATP synthesis in skeletal muscle suggesting an inherited defect in oxidative phosphorylation that may predispose these individuals to the development of diabetes. This defect may be present in the β-cell as well, disrupting glucose induced insulin release. 4) Overexpression of the mitochondrial uncoupling protein (UCP-2) in rat islets

disrupts glucose induced insulin secretion by dissipating the mitochondrial proton gradient and uncoupling oxidation and ATP production [119]. Conversely, genetic deficiency of UCP-2 or targeted knockout of the gene increases $[ATP]_i$ (decreases $[ADP]_i$) and enhances insulin secretion [120] [121]. Moreover, UCP2-expression is regulated by PPARγ and can be induced by chronic exposure of rat islets to glucose and fatty acids, an environment often found in pre-diabetic and diabetic patients [122]. In humans β-cells UCP-2 is also upregulated by hyperglycemia [123]. 5) Peroxisome proliferator receptor γ coactivator 1α (PGC-1α), a transcriptional co-activator important in skeletal muscle mitochondrial biogenesis, is present at lower protein levels in the skeletal muscle of type II diabetics as well as individuals at risk for diabetes [116] [124]. Interestingly, PGC-1α expression is higher in the β-cells of several animal models of type II diabetes [125]. Subsequent experimental overexpression of PGC-1α in otherwise normal islets resulted in inefficient ATP production, elevated basal secretion of insulin, and markedly reduced glucose induced insulin secretion. This effect is reminiscent of that seen in β-cells chronically treated with fatty acids [122]. Indeed, Zhang et al recently demonstrated that PGC-1α upregulation is induced by hyperlipidemia and not hyperglycemia [126]. 6) In association with the increased levels of circulating free fatty acids and skeletal muscle accumulation of triglycerides, there is a decrease in the activity of enzymes responsible for lipid oxidation in the mitochondria of diabetic patients [127] [128] 7) Aging, a risk factor for type II diabetes, is correlated with a decrease in mitochondrial function [129] and an increase in the production of reactive oxygen species (ROS) in the mitochondria [130]. Relative to other cell types, β-cells contain a particularly low level of protective enzymes (catalase and superoxide dismutase) against

free radicals, which may render islets more susceptible to ROS induced damage resulting in abrogated glucose induced insulin secretion. 8) An autosomal dominant form of diabetes termed maturity onset diabetes (MODY) is linked to mutations in the transcription factors hepatocyte nuclear factors HNF-4α and HNF-1α, which appear important for normal mitochondrial metabolism [131] [132]. 9) A decrease in the β-cell mass, perhaps via apoptosis is thought to contribute to the development of diabetes, and mitochondria are key controllers of programmed cell death [119]. All of these alterations to mitochondrial function could be potentially detrimental to glucose-induced decreases in free ADP and insulin secretion.

Alternatively, diabetic and pre-diabetic β-cell mitochondria may function properly but high levels of circulating free fatty acids due to obesity become the dominant substrate for mitochondria. Preferred β-oxidation of fatty acids would then clamp mitochondrial produced ADP at a high level. A glucose challenge on top of this high baseline of fatty acids is therefore unable to induce a sufficient decrease in ADP to a concentration where the K^+_{ATP} channel could be modulated. The K^+_{ATP} channel is thus chronically opened, preventing insulin secretion and inducing hypoglycemia. Specifically, chronic fatty acid induced UCP-2 upregulation would keep ADP levels high by dissipating the glucose induced mitochondrial gradient and preventing efficient ATP synthesis. A glucose challenge under these conditions would be ineffective in eliciting insulin secretion. If this is the case, the upregulation of PGC-1α observed in islets of diabetic animals may be an attempt by the β-cell to increase mitochondrial density in order to sufficiently decrease ADP and secrete adequate insulin. All of these proposals are testable with our ^{31}P-NMR bioreactor system.

Bioreactors

The development of a viable bioreactor is also a significant outcome of this research that may prove useful for future ^{31}P-NMR evaluations of insulin secreting cells as well as alternative cell types. More importantly, these bioreactors could be valuable tools for β-cell transplantations and treatment of type I and type II diabetes. One problem that has hindered the therapeutic use of islet transplantation is the inability of researchers to evaluate the integrity of insulin secreting cells in animal models before and after transplantation. Our bioreactor could easily be examined by ^{31}P-NMR before implantation and then be removed from the experimental animal and re-examined to determine the functional capacity of the cells. An additional hindrance to implantable bioreactors is the difficulty of providing adequate oxygen and nutrients to the non-vascularized tissue inside the bioreactor. The small diameter of our bioreactors could address this problem. Our cells received adequate diffusion of oxygen and nutrients as evidenced by the integrity of ATP and Pi levels over several hours in the magnet. Further, the membranous tubing used to encapsulate the cells could be coated with a factor such as VEGF to induce vascularization of the bioreactor [133]. If implantation of the bioreactor proves feasible and the cells remain functional and therapeutically useful over a significant period of time, this bioreactor could also be used to gain insight into the etiology of diabetes. Bioreactors with healthy β-cells could be implanted into different diabetic mouse and rat models, and the effect of the diabetic environment could be evaluated at different time points. Based on conclusions reached using INS-1 cells, we

would expect that the diabetic milieu would compromise the ability of β-cell mitochondria to adequately and efficiently lower ADP to induce insulin secretion.

Conclusion

Glucose induced insulin secretion appears to be associated with a decrease in free $[ADP]_i$ without any significant changes in $[ATP]_i$ The mechanism relating this mitochondrially regulated metabolic change to 1st and 2nd phase insulin secretion is proposed as follows: 1) Immediately following a glucose challenge, $[ADP]_i$ begins to decrease thus promoting closure of membrane K^+_{ATP} channels and β-cell depolarization. 2) Voltage-gated Ca^{2+} channels are activated, increasing $[Ca^{2+}]i$ to trigger 1st phase insulin secretion of docked and primed insulin granules. 3) As 1st phase insulin reaches a peak and begins to decline, $[Ca^{2+}]_i$ remains elevated and sustained mitochondrial glucose metabolism continues to drive a decrease in free [ADP]i. 4) This prolonged decrease in free $[ADP]_i$ promotes docking and priming of the reserve granules and RR pool to replenish the IR pool of insulin granules for 2nd phase insulin secretion. Glucose induced acidification of the intracellular environment enhances this replenishment process. 5) The new steady state minimum of $[ADP]_I$, reached 48 minutes after a glucose challenge, establishes the maximal steady state rate 2nd phase insulin secretion by determining the rate of IRP replenishment. 6) Insulin secretion is terminated following the removal of a glucose challenge as ATPase activity increases free [ADP] back towards its basal concentration, thus preventing IRP replenishment and re-opening membrane K^+_{ATP} channels. This proposed control of both 1st and 2nd phase insulin secretion by mitochondrially regulated

ADP has numerous implications for understanding normal and pathological glucose induced insulin secretion that remain to be experimentally validated.

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