Insulin-Stimulated Phosphate Transport and ATP Synthesis in Skeletal Muscle

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INTRODUCTION

Insulin is a very potent anabolic hormone, the action of which is important for us to understand. It is the mainstay of hyperglycemic control in diabetic patients. Research on insulin action has been focused primarily on glucose transport and energy metabolism, for good reason. There are, however, other concomitant effects of insulin action which are important to understand. One of these is the effect that insulin seems to have on the redistribution of orthophosphate (P_i) from the plasma to the intracellular spaces of the liver and skeletal muscles. The hypophosphatemic effects of insulin administration in the setting of diabetes were documented over 70 years ago [1], yet the transporters and mechanisms involved in mediating this effect have yet to be described. Understanding this phenomenon is important, as its characterization may lead to insights regarding the control of mitochondrial oxidative function which is becoming increasingly implicated in the pathogenesis of insulin resistance and type 2 diabetes mellitus, one of the world's most devastating diseases.

SPECIFIC AIMS

1. IDENTIFY THE TRANSPORTERS MEDIATING INSULIN-STIMULATED INORGANIC PHOSPHATE TRANSPORT IN SKELETAL MUSCLE.

Examination of the current literature, in conjunction with database searches, can be used to create a list of putative P_i transporters. The presence/absence of transporter mRNA can then be screened for in our systems of interest (L6 rat myoblast cell culture and mouse soleus muscle) using real-time polymerase chain reaction (RT-PCR) based methods. Where present, further characterization can be undertaken by Western Blotting analysis. The participation of candidate transporters in insulin-stimulated phosphate transport can be verified by conducting phosphate transport studies following siRNA knockdown of protein expression in the L6 cell culture system. We hypothesize that the NaPi type-III cotransporters PiT-1 and PiT-2 are the most likely candidates.

2. DETERMINE THE MECHANISMS OF ACTION OF INSULIN-STIMULATED INORGANIC PHOSPHATE TRANSPORT IN SKELETAL MUSCLE.

Insulin-stimulated phosphate transport has been previously demonstrated in L6 cells and soleus muscle strips [2, 3] and seems to be a mediated by a process independent of protein synthesis [4]. We hypothesize that an insulin-stimulated signal transduction cascade acting through phosphoinositide 3-kinase (PI3K) is responsible for the activation of P_i transport, possibly through the recruitment of transporters to the plasma membrane as previously suggested [3]. The components of this signaling pathway can be manipulated in L6 cells by genetic and pharmacological means, and their respective roles in insulin-stimulated P_i transport discerned.

3. DETERMINE THE REGULATORY ROLES OF INORGANIC PHOSPHATE TRANSPORT ON ATP SYNTHESIS IN SKELETAL MUSCLE.

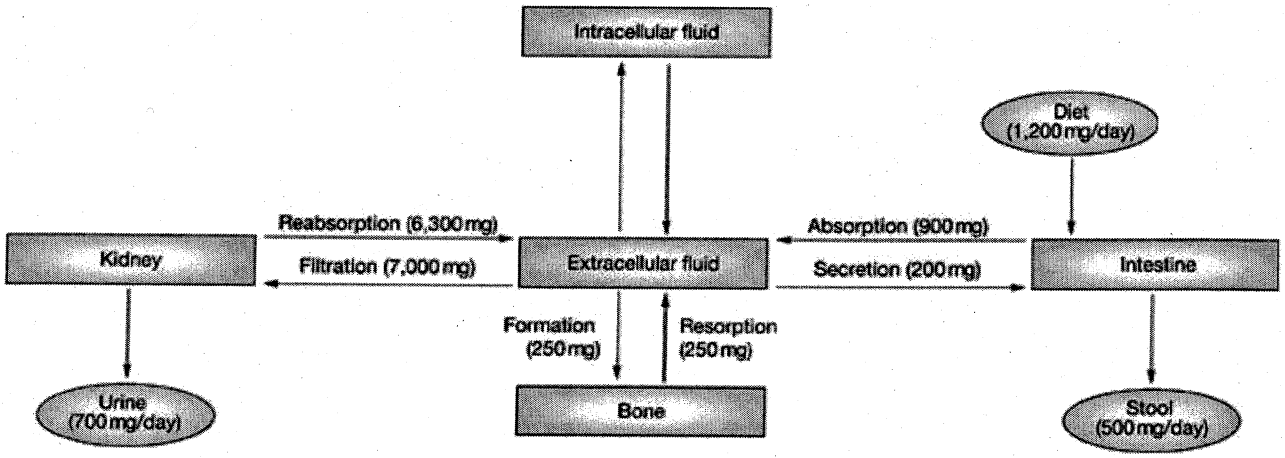
Insulin-stimulated ATP synthesis is blunted in insulin-resistant individuals [5]. These individuals also exhibit blunting of insulin-stimulated P_i transport [5]. We hypothesize that a causal relationship exists between these two observations in that insulin-stimulated ATP synthesis is, at least in part, regulated by the increase in P_i transport induced by insulin stimulation. ATP levels and synthetic rates can be assessed using biochemical assays and ^{31}P magnetic resonance spectroscopy (^{31}P MRS), respectively.

BACKGROUND AND SIGNIFICANCE

The stimulation of skeletal muscle with insulin results in numerous effects surrounding energy metabolism which have been studied extensively. A lesser-investigated effect of insulin on skeletal muscle is its ability to stimulate that transport of inorganic phosphate (P_i) into myocytes in a sodium-dependent fashion [2, 3]. Although there is some evidence to support the hypothesis that type III NaPi cotransporters may be responsible for this effect [6], no conclusive evidence has been reported. Moreover, there has been little, if no, report of the mechanisms acting to effectuate insulin-stimulated phosphate transport in skeletal muscle. Understanding this process is important because the elucidation of the mechanisms involved may shed some light on the question of mitochondrial oxidative function. Not only is P_i a substrate for oxidative phosphorylation, but it also appears to be a signaling molecule in the process [7]. With mitochondrial dysfunction becoming increasingly implicated in the development of insulin resistance, understanding the control of mitochondrial oxidative function may hold the key to understanding, and perhaps treating, insulin resistance.

Phosphate Homeostasis

The regulation of phosphate homeostasis is a process involving multiple organ systems. Phosphate is essential for numerous aspects of cellular function including energy metabolism (glycolysis, glyconeogenesis, ATP synthesis, phosphocreatine synthesis), skeletal mineralization, nucleic acid biosynthesis, membrane phospholipids synthesis, and protein phosphorylation. The schematic below [8] illustrates the fate of ingested phosphate and the distribution of phosphate amongst the body's various pools. Long-term P_i regulation involves the kidneys and intestine. Under conditions where P_i intake is low, the intestine increases its absorptive efficiency and the kidneys do their part by increasing P_i transport and reducing urinary P_i loss. Certain physiologic conditions, such as growth, pregnancy, and lactation, are associated with an increased need for P_i, and hence an increase in P_i absorption and retention. Seeing as most of the P_i which is absorbed on a daily basis is excreted in the urine, P_i balance and P_i plasma concentrations are largely dependent on renal regulatory mechanisms. The type II NaPi cotransporters, for example, are largely responsible for renal P_i reabsorption, 70-80% of which happens in the proximal tubule.



Dysregulation in the fluxes shown in the schematic can lead to hypophosphatemia. There are thought to be three potential mechanisms at play. There may be a decreased intake of P_i, a redistribution of P_i from the plasma to other pools, or an increase in urinary excretion of P_i. Of particular interest in our discussion is the potential for hypophosphatemia to result from the

administration of insulin or from the release of endogenous insulin during refeeding in diabetic patients. It is thought that that principle mechanism at play in such scenarios is the redistribution of P_i from the plasma to the liver and skeletal muscles. Although hypophosphatemia rarely results from such treatments, the clinical consequences can be significant, and include rhabdomyolysis, hemolysis, leukocyte dysfunction, respiratory failure, impaired myocardial performance, diabetic ketoacidosis, and perturbed central nervous system function [8].

Phosphate Transporters

So far, at least three different types of sodium-phosphate cotransporters have been cloned and characterized, and are denoted as types I (SLC17), II (SLC34), and III (SLC20). Type I NaPi cotransporters are expressed primarily in the kidneys, liver and brain [9], and also have chloride channel activity [10]. There are three type II cotransporters, denoted IIa, IIb, and IIc. The type IIa cotransporter is largely renal-specific [11] while the type IIb is thought to be primarily responsible for intestinal P_i uptake [12]. The type IIc is thought to be a growth-related NaPi cotransporters expressed renally during the weaning period [13]. Thus far, there is no evidence to suggest that the type I and II cotransporters are expressed in skeletal muscle, and are thus unlikely to be involved in insulin-stimulated phosphate transport in this tissue.

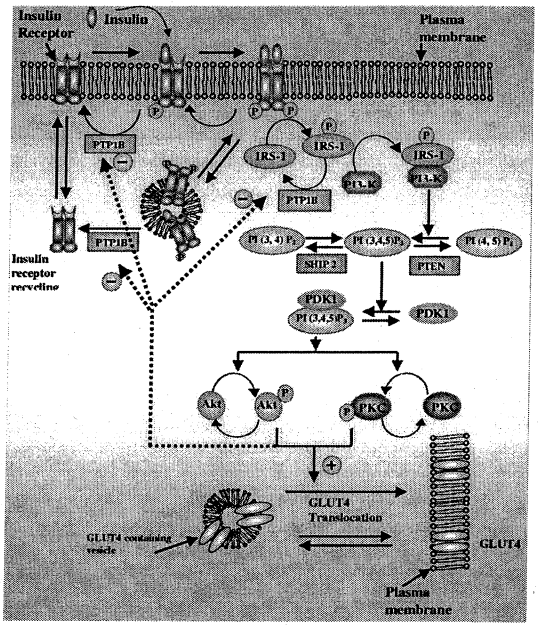
There are currently two members of the NaPi-III cotransporter family, PiT-1 (SLC20A1) and PiT-2 (SLC20A2). These are both widely expressed transmembrane proteins that were first discovered as retroviral receptors [14, 15] and only later shown to be NaPi cotransporters [16, 17]. PiT-1 was originally termed the gibbon ape leukemia virus receptor 1 (Glvr-1) and PiT-2 the rat amphotropic leukemia virus receptor 1 (Ram-1). Extensive studies have been carried out on these receptors/cotransporters, and their functional characteristics have been documented. Overexpression in *Xenopus* oocytes has shown that transport of phosphate is not only sodium dependent, but is electrogenic with a stoichiometry of two sodium ions for every phosphate ion cotransported [16, 18]. In *Xenopus* oocytes, the K_m for P_i was 24.1±5.5μM for human PiT-1 [16], 25.3±6.0 μM for rat PiT-2 [16], and 44.0±24.0 μM for mouse PiT-2 [18].

The presence and physiological function of PiT-1 and PiT-2 have been studied in a variety of tissues and organs, including the parathyroids, arteries, the kidneys, intestine, and bone. A review of these findings is beyond the scope of this prospectus. Of note, however, is the relatively little work that has been carried out in skeletal muscle with regards to PiT-1 and PiT-2. One notable study examined P_i transport and its relation to PiT-1, and found that there was a seemingly linear relationship between P_i uptake and PiT-1 protein expression when examining a spectrum of muscles including cardiac, gastrocnemius, and soleus muscle [6]. This apparent dependence of P_i uptake on PiT-1 expression places PiT-1 as a prime candidate for playing an important role in mediating P_i uptake in skeletal muscle.

Insulin Signaling

The pictured schematic is a simplified representation of the insulin signaling network, or pathway [19]. This pathway is also involved in cellular processes such as protein synthesis and cell survival. The most dramatic and clinically relevant effects of insulin resistance, however, are seen in the inhibition of the pictured pathway, leading to decreased glucose uptake and hyperglycemia. Given the rapidity of insulin-stimulated phosphate uptake observed clinically and experimentally, it is reasonable to believe that the uptake of phosphate may be regulated in a similar fashion to that of glucose, meaning that uptake is not dependent on novel protein synthesis, but rather on the translocation of ready-made transporters to the plasma membrane.

Such a hypothesis is further supported by our observations in offspring of type 2 diabetic parents. Not only were the offspring insulin-resistant with reduced whole-body glucose metabolism, but their capacity for insulin-stimulated phosphate transport was diminished, as well [5]. The concurrence of these diminished capacities is supportive of the idea that both phosphate and glucose uptake may be executed by the same effectors.



Insulin Resistance, Type II Diabetes Mellitus, and Mitochondria

Type 2 diabetes is a disease of pandemic proportions responsible for staggering amounts of morbidity and mortality, causing 4 million deaths per year [20] and costing over $132 billion in the U.S. alone in 2002 [21]. Although the precise pathogenetic mechanisms of diabetes remain unknown, it is widely agreed upon that a state of insulin resistance precedes that of frank diabetes and loss of glycemic control. Moreover, a reproducible pattern of disease known as metabolic syndrome, or syndrome X, is becoming increasingly common. This includes the concurrence of insulin resistance, central obesity, dyslipidemia, and hypertension. One school of thought describes diabetes and metabolic syndrome as a dysfunction in energy metabolism. In the case where more energy is consumed than is expended, the body can be said to be in positive energy balance. The human body uses anabolic systems, such as insulin, to create stores of energy that can be called upon in a time of need where calories are in short supply. Recent history has seen a dramatic shift in caloric availability that, in conjunction with dietary habits in excess of necessity, has led to an increasing proportion of the population spending time in perpetual positive energy balance. The physiologic long-term storage of calories in the human body is adipose tissue, whose capacity for lipid storage is finite. Interestingly, as positive caloric balance is maintained in the face of a calorie storage pool filled to capacity, the body does not stop producing the high-caloric-content molecules, lipids. Instead, lipid storage continues and spills over into a pathologic state where lipid storage occurs at non-adipose sites. At this point, both dyslipidemia and obesity are at play. It has been shown that extra-adipose lipids will accumulate in the liver and skeletal muscles of such individuals, and can lead to the serine phosphorylation of key insulin signaling pathway intermediates, possibly inhibiting their action and causing insulin resistance.

The theoretical string of events proposed above can be thought of as a plausible explanation for cases where excessive and constant positive caloric balance is maintained. But what does this have to do with young, lean, insulin resistant individuals? These individuals have also been shown to have increased intramyocellular lipids (IMCLs). The real question, then, becomes: why do they store IMCLs if there is room in their adipose tissues, even if such IMCL accumulation can have pathologic effects and lead to insulin resistance? Increasingly, dysfunctional mitochondria have been placed on the chopping block. Lipids are oxidizeable fuels that mitochondria can use to make ATP. In a situation where mitochondria are present in inadequate numbers or are dysfunctional, one can imagine the inappropriate accumulation of mitochondrial fuel, or lipids, within the affected cells. As previously described, such an

accumulation of intramyocellular lipids (IMCLs) can lead to blunting of the insulin signaling pathway and insulin resistance.

Insulin resistant individuals have also demonstrate reduced mitochondrial oxidative function [22] as well as reduced mitochondrial density [23]. The resulting hypothesis unifying these findings is one where deficiencies in mitochondrial oxidative function (be it through reduced density or secondary to dysfunction) result in elevated IMCL concentrations leading to the aberrant serine/threonine phosphorylation and inhibition of key insulin signaling mediators, manifesting as insulin resistance in skeletal muscle. Evidence in support of this hypothesis would suggest that increasing mitochondrial oxidative function could reverse insulin resistance by melting away IMCLs. Interestingly, however, an increase in cellular mitochondrial content is not sufficient to protect against the development of insulin resistance in a transgenic mouse model overexpressing PGC1α (a transcriptional coactivator of mitochondrial biogenesis) with twice the amount of mitochondria as WT mice. The crux of the issue seems to lie at the regulation of mitochondrial oxidative function, which we do not yet understand.

Mitochondrial ATP Synthesis

Skeletal muscle energy metabolism is enormously complicated, given the number of intermediary metabolites and the physiological pools into which they are subdivided. The simple conceptual model of mitochondria making ATP on an as-needed basis in response maintaining the ATP/ADP ratio at a predetermined level, although at times convenient, cannot account for the exhibited dependence of ATP synthesis on phosphocreatine (PCr) and P_i, for example. Recent studies have shown that P_i may act as a cytosolic signaling molecule in the regulation of oxidative phosphorylation in that it was shown to modulate the formation of ATP by the F_1F_0-ATPase in heart and skeletal muscle mitochondria [7]. One study has determined that there are at least two interconvertible kinetic modes for ATP synthesis by the F_1F_0-ATPase, and that under conditions where free energy for synthesis is readily available, the apparent K_m values for ADP and P_i are relatively high at approximately 50-100 μM and 2.0 mM, respectively [24]. The intracellular P_i concentration in skeletal muscle is somewhere in the range of 2-4 mM [25, 26], leaving room for the possibility that intracellular P_i concentration can play a role in the modulation of ATP synthesis at the level of the F_1F_0-ATPase.

^{31}P Magnetic Resonance Spectroscopy and Saturation Transfer

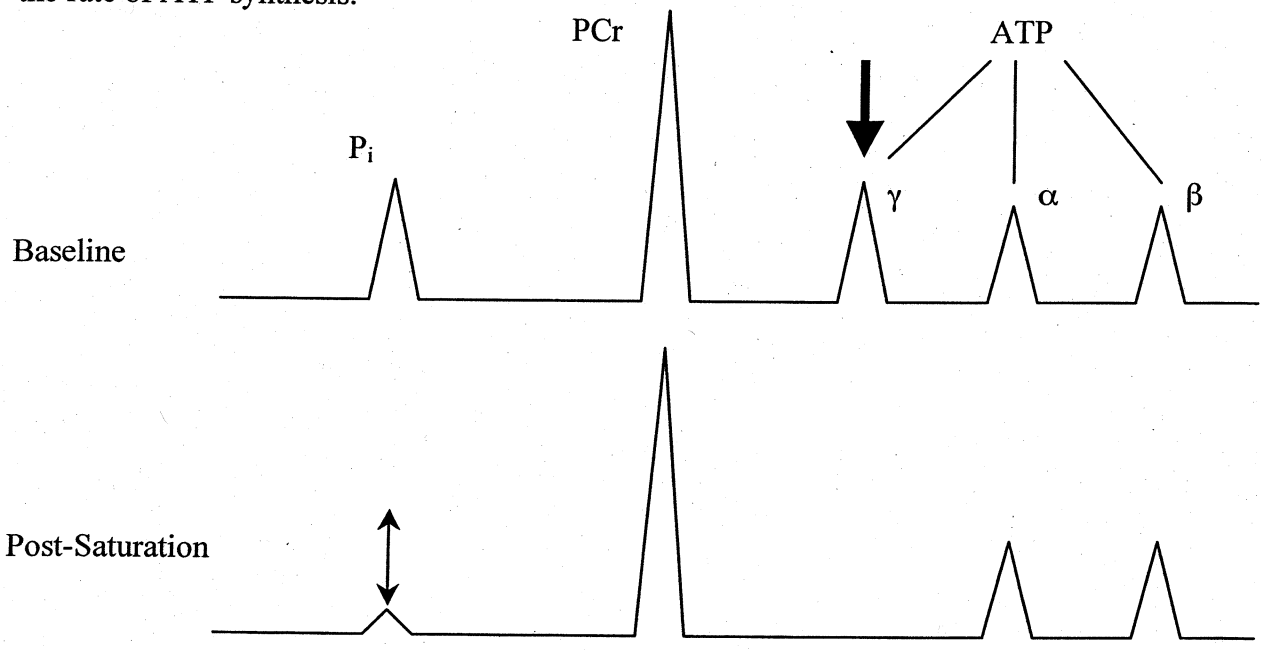
It is beyond the scope of this prospectus to describe the fundamental physical principles involved in magnetic resonance spectroscopy (MRS), or the complex mathematics that are required to transform the data obtained into a meaningful format. A brief explanatory discussion of the saturation transfer principle, however, is in order. The equilibrium equation below describes the synthesis of ATP from ADP by the mitochondria. This is a reversible reaction.

$$ADP + P_i \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ATP$$

The equation below describes the measurement of the P_i peak in the ^{31}P spectrum, where M stands for the total magnetic signal, M_o for the magnetic signal from free P_i, T_1 for longitudinal relaxation time, and k_f and k_r are the equilibrium constants for the forward and reverse reactions of the equilibrium between ATP and ADP. The arrow on the baseline spectrum indicates which signal is being magnetically suppressed (gamma phosphate of ATP).

$$\frac{dM}{dt} = \frac{dM_o}{T_1} - k_f d[P_i] + k_r d[ATP]$$

The first step is to obtain a baseline spectrum and to determine the amount of intracellular P_i, represented by the denoted peak in the spectrum below, which is said to be in exchange with the gamma phosphate of ATP, as depicted in the equilibrium shown above. One must then come to the realization that this peak is composed of the summation of contributions depicted in the equation above. The first term describes the contribution from the phosphate nuclei in the free P_i form. This amount, however, is modified in two ways, as described by the equilibrium equation. The second term in the above equation describes the amount of P_i that is lost into ATP, and the third term describes the amount of P_i that is derived from the degradation of ATP. The next step is to hit only the nuclei of the gamma phosphate of ATP with an electromagnetic pulse (large downward arrow) that will render them magnetically invisible (known as saturation), and another spectrum is obtained (post-saturation). At this point, something very interesting and useful happens to the spectrum. You will note, by comparing the post-saturation spectrum below to that of the baseline, that two things have changed. The first, and more obvious, is that the peak which represented the gamma phosphate of ATP is gone. This is due to the magnetic saturation described above. The second, and more subtle, is that the P_i peak is somewhat smaller. Why? Let us be reminded of the fact that P_i is always in exchange with ATP, and of the terms of the equation shown above. Here is where it all comes together. Under the described saturating conditions, the third term becomes equal to zero. There is no visual contribution of ATP hydrolysis into the P_i peak. However, keep in mind that an equilibrium is in effect, meaning that the rates of the forward and reverse reactions are equivalent. As a result, any P_i that becomes incorporated into ATP will become magnetically silenced and once returned to the P_i pool, will be effectively invisible, hence a reduction in the apparent amount of P_i. Therefore, the difference between the magnitudes of the P_i peaks in these spectra (double headed arrow) can be related to the rate of ATP synthesis.



Model Systems

We intend to make use of two model systems in our investigations. One is the L6 cell line, and the other is viable, intact, mouse soleus muscles. The L6 cell line is a rat myotubule line that was established in 1968 [27]. It is a cell line that proliferates in a myoblastic form which can be induced to differentiate into myotubules, a form more closely reminiscent of skeletal muscle tissue. The advantages in studying cell lines are that they can be relatively easily manipulated and they can be perturbed genetically in ways that whole tissues or animals cannot. The principal disadvantage, however, is their potential irrelevance to what happens *in vivo*. To circumvent this shortcoming, we plan to repeat our results obtained in cell culture in live, oxygenated, superfused mouse soleus muscles. Given that tissue viability sustained by superfusion is a diffusion-limited phenomenon, rat soleus muscles cannot be used in their entirety as they are too thick and would yield apoxic and necrotic cores during experimentation, possibly confounding our experimental results. Mouse soleus muscles, on the other hand, are small and thin enough to be adequately oxygenated by superfusion.

The group led by Sir George Radda at the University of Oxford has done a significant amount work in the study of phosphate transport in skeletal muscle using L6 cell culture and superfused rat soleus muscles, as well as a variety of other systems. They have demonstrated, both in L6 cells and in a rabbit skeletal muscle sarcolemma/t-tubule vesicle preparation, that P_i uptake is sodium dependent [2]. In addition, in rat soleus muscle strips and the mouse G8 myoblast cell line, P_i uptake has been shown to be stimulated both by insulin and IGF-1 [3]. We will establish these experimental model systems in our laboratory to further examine the physiological and cellular mechanisms underlying insulin-stimulated phosphate transport in skeletal muscle.

PRELIMINARY RESULTS

AIM 1: IDENTIFY THE TRANSPORTERS MEDIATING INSULIN-STIMULATED INORGANIC PHOSPHATE TRANSPORT IN SKELETAL MUSCLE.

The most likely transporter candidates mediating this phenomenon are the previously described NaPi cotransporters, types I, II, and III. Database searched have also revealed several putative phosphate transporter proteins in the SLC25 family.

Family	*Gene*	*Protein*	*Alias*	*Accession ID*	*Tissue Expression*
Type III	SLC20A1	PiT-1	Glvr-1	NM_005415	Sk. muscle, marrow, kidney, intestine, parathyroid, lung, liver
	SLC20A2	PiT-2	Ram-1	NM_006749	Cardiac and skeletal muscle, brain, kidney, lung
Type II	SLC34A1	NPTIIa		NM_003052	Kidney, lung
	SLC34A2	NPTIIb		NM_006424	Intestine, lung
Type I	SLC17A1	NPT1		NM_005074	Kidney, liver
	SLC17A2	NPT2/3		NM_005835	Liver, lung, muscle, brain
	SLC17A3	NPT4		NM_006632	Kidney
	SLC17A6	DNP1		NM_020346	Brain, liver
	SLC17A7	BNP1		NM_020309	Brain
Mitochondrial Transporter Family	SLC25A3	PHC	PTP	NM_002635	Brain, heart, skeletal muscle
	SLC25A23	APC2		NM_024103	Brain, heart, skeletal muscle, prostate, kidney, lung
	SLC25A24	APC1		NM_013386	Heart., prostate, kidney, lung
	SLC25A25	SCaMC-2		NM_052901	Brain, muscle, pancreas, prostate

AIM 2: DETERMINE THE MECHANISMS OF ACTION OF INSULIN-STIMULATED INORGANIC PHOSPHATE TRANSPORT IN SKELETAL MUSCLE.

System Validation, L6 Myoblast Cell Culture

L6 cells are a rat skeletal muscle cell line that can be induced to differentiate into myotubules when cultured under reduced-serum conditions (2% FBS as opposed to the typical 10%). Upon differentiation, cultured cells assume a more organized and spindle-like morphology, reminiscent of bands of elongated muscle fibers. Presumably, differentiation will render cell cultures more physiologically akin to skeletal muscle. Previous studies using L6 cultures for the study of phosphate transport, however, exclusively utilized undifferentiated cultures [2]. We examined cultures in both states of differentiation to determine which model would be better suited for the study of insulin-stimulated P_i transport. The results shown in Figure 1 indicate that the undifferentiated cultures show greater changes in insulin-stimulated P_i transport. They also show that in the differentiated state, there seems to be a PI3K-dependent increase in basal P_i transport, which can confound the examination of insulin stimulated increases in P_i transport that are effected via a PI3K-dependent pathway. As a result, we will use the undifferentiated L6 cell culture as our model system in future studies. This has the added advantage of eliminating the need to differentiate cells with 2% FBS-supplemented media, which could take longer than one week, thus eliminating further experimental variables from culture preparation.

Previously published reports examining insulin-stimulated P_i transport indicate that the majority of this transport is sodium-dependent [3], which is demonstrated in Figure 2. Unfortunately, there was only a mild insulin-stimulated change in P_i transport in this study, and we plan to repeat this experiment to verify these results. Figure 3 illustrates the effect of incubation time on the observed rate of insulin-stimulated P_i transport. Based on these results, we plan to use an incubation time of 10 minutes for our future studies, as this time point allows for good discrimination between basal and insulin-stimulated rates, and is also compatible with our experimental protocol design.

The L6 culture model was also evaluated in terms of the insulin signaling pathway, which we suspect to be very much involved in the processes we aim to characterize. Western Blot analysis, shown in Figure 4, indicates that the insulin signaling pathway is indeed intact up to the level of Akt phosphorylation in L6 cells. Akt phosphorylation is stimulated by insulin in a dose-dependent manner and completely blocked by inhibition of the effective kinase, PI3K, by wortmannin.

System Validation, Mouse Soleus Muscle Strips

Although L6 cultures provide a reproducible and readily perturbable system in which to study insulin-stimulated P_i transport, the reproduction of obtained results in intact muscles would be of significant value in bringing physiologic relevance to our findings. Live, intact mouse soleus muscles can be dissected and kept viable by static incubation in oxygenated buffer. Although not readily perturbed by genetic means, these preparations can be subjected to pharmacological treatment and assayed for phosphate transport in a manner similar to the L6 model. Figure 5 indicates that such preparations exhibit insulin-stimulated P_i transport, and Figure 6 demonstrates the integrity of the insulin signaling pathway in oxygenated mouse soleus muscles up to the level of Akt phosphorylation.

AIM 3: DETERMINE THE REGULATORY ROLES OF INORGANIC PHOSPHATE TRANSPORT ON ATP SYNTHESIS IN SKELETAL MUSCLE.

Figure 7 illustrates an example of a ^{31}P MRS saturation transfer experiment. Spectrum A is saturated with a control pulse set a frequency of 2100 Hz, while spectrum B is saturated with a pulse targeted at the resonant frequency of the gamma phosphate of ATP, -897 Hz (downward arrow). Despite the high noise level in these spectra, some attenuation of signal (bracketed region) can be observed in the downfield peaks at approximately 1, 3, and 5 ppm. The exact identity of these peaks remains to be determined. These spectra are shown for illustrative purposes and as evidence that this technique may be successfully applied to the question at hand.

RESEARCH DESIGN AND METHODS

AIM 1: IDENTIFY THE TRANSPORTERS MEDIATING INSULIN-STIMULATED INORGANIC PHOSPHATE TRANSPORT IN SKELETAL MUSCLE.

Rationale

The phenomenon of insulin-stimulated phosphate transport has been previously described both at the bench and the bedside [1, 2]. The key transporters mediating this transport, however, are yet to be identified. The most likely candidates are the previously described NaPi cotransporters. Our initial investigative list contains these proteins, as well as several members of the mitochondrial transporter family SLC25 (Table 1).

Hypothesis

Based on previously published results regarding transporter tissue expression and subcellular localization patterns, as well as our own preliminary studies, we hypothesize that PiT-1 and PiT-2 are the phosphate transporters most likely to be the key mediators of insulin-stimulated phosphate transport in skeletal muscle [6].

Methods

Working from our list of thirteen putative transporters, shown in Table 1, we will systematically screen them for relevance. The first step will be to test for the expression of the genes in question in our two model systems, rat L6 cell cultures and mouse soleus muscles. Gene expression will be assessed by mRNA quantification using RT-PCR. Oligonucleotide probes will be custom designed using the Primer3 algorithm from the Whitehead Institute, and ordered from Integrated DNA Technologies, Inc., Coralville, IA. RT-PCR will then be carried out using the SYBR Green amplification detection system. Lack of gene expression will eliminate the need for further testing of those transporters whose mRNA is absent. Cells and tissues where expression of each gene has been previously reported will be used as positive controls. In cases where pertinent mRNA is present, protein expression levels can be evaluated by Western Blot analysis. The necessary antibodies are commercially available and tissues/cells that have been previously reported to express the transporters of interest will be used as positive controls. The most conclusive evidence, however, of transporter relevance will be obtained by transporter knockdown using siRNA techniques, followed by an insulin-stimulated ^{32}P uptake assay. siRNAs will obtained from Dharmacon of Chicago, IL, and non-specific siRNA will be used to control for non-selective knockdown. Loss of insulin-stimulated P_i uptake concomitant with transporter knockdown will confirm the effective role for that given transporter.

Expected Results

Based on previously published expression profiling data, we expect that a number of these transporters will not be significantly expressed in our experimental systems (L6 cells and mouse soleus). Furthermore, we predict that the knockdown of PiT-1 and PiT-2 will show the most dramatic reduction of insulin-stimulated ^{32}P uptake.

Limitations and Alternatives

Of course, our list of putative transporters is not exhaustive and likely not all-inclusive; it is merely a sample of the most likely candidates. If no critical transporters can be identified in this fashion, our approach will require some revision, perhaps using broader, more inclusive screening techniques to identify the relevant transporters. One anticipated limitation to the methods outlined above is the availability of the pertinent antibodies or siRNA molecules for Western Blot analysis and knockdown experiments, respectively. An alternative/corroborative approach to assessing transporter relevancy would be to conduct the same ^{32}P uptake assays while overexpressing the transporter candidates in L6 (or other) cell cultures.

AIM 2: DETERMINE THE MECHANISMS OF ACTION OF INSULIN-STIMULATED INORGANIC PHOSPHATE TRANSPORT IN SKELETAL MUSCLE.

Rationale

Previously published results [3], as well as our preliminary findings (Figure 3), indicate that insulin-stimulated phosphate transport occurs on the order of minutes and not hours, suggesting that this process is not dependent on insulin-stimulated protein synthesis. There has been no description, to the best of our knowledge, of relevant signaling pathways or second messengers, nor of the fate of transporters and their mechanisms of recruitment/activation.

Hypothesis

We hypothesize that insulin-stimulated phosphate transport is mediated by the insulin signaling pathway via PI3K action, and may involve the translocation of phosphate transporters to the plasma membrane in response to insulin stimulation.

Methods

We have validated a method of quantifying phosphate uptake using radiolabeled inorganic phosphate ($H_3{}^{32}PO_4$). L6 cell cultures can be perturbed in various ways and stimulated with insulin to induce phosphate transport, which can be assayed for by measuring the rate of ^{32}P uptake under different conditions. The most direct assessments of PI3K involvement in this process can be made by pharmacologically inhibiting PI3K with small molecule inhibitors, such as wortmannin and LY294004. Alternatively, PI3K can be knocked down by siRNA followed by assessment of ^{32}P uptake as previously described. Another possibility is to overexpress a constitutively active form of p110 (catalytic subunit of PI3K) in L6 cell cultures. In addition to L6 cell culture, the small molecule inhibitor experiments can be conducted in mouse soleus strips as well, for added physiological relevancy and robustness. Moreover, other components of the insulin signaling pathway such as Akt, which classically acts downstream of PI3K, can be studied to add supporting evidence to the hypothesis that insulin-stimulated phosphate transport is PI3K-dependent. The small molecule inhibitor KP372-1 [28] has been shown to inhibit

phosphorylation by Akt. The role of Akt can, thus, be assessed using this inhibitor followed by phosphate uptake analysis.

In addition to examining the signaling events involved in insulin-stimulated phosphate transport, we also plan to investigate what is happening at the level of the transporters. Unless insulin signaling acts in some way to actually alter the characteristics of the phosphate transporters, i.e. K_m, one can assume that the increased rate of phosphate transport is due to a proportional increase in V_{max}. When the K_m and the concentration of the transported substrate are held constant, an increase in V_{max} can only be effectuated by a proportional increase in the concentration of active transporters. What does that mean in *this* case? Is *this* a phenomenon with a mechanism of action similar to that of insulin-stimulated glucose uptake mediated via GLUT4? GLUT4-containing vesicles near the cell surface fuse with the plasma membrane upon insulin stimulation and effectively increase the number of active glucose transporters on the cell surface, effectively increasing V_{max} for glucose uptake. There are a variety of methods that we can employ to investigate this hypothesis, each with their host of hurdles and limitations.

One straightforward approach to studying transporter translocation is to employ an antibody targeted to an extracellular epitope of the transporter in question. With an attached cell line like L6 cells, an ELISA approach can be employed where an HRP-conjugated secondary antibody is used to detect and quantify the presence of a primary antibody directed against an extracellular epitope of our transporter of interest. This assay can be performed on numerous wells of cells at a time and under different conditions, including pharmacologic and genetic perturbations. Immunofluorescent assessment of transporter translocation can be assessed by using a similar set-up as described above, but with the replacement of HRP with a fluorescent moiety. Another technique commonly used to assess protein translocation to the plasma membrane makes use of surface biotinylation. Following cell-surface biotinylation, the cells are washed and lysed. A fraction of this total lysate is retained. The remainder is incubated with streptavidin-coated agarose beads, which are then washed thoroughly. The sample is then eluted from the beads with SDS-containing buffer and separated by PAGE, followed by Western Blotting with the appropriate antibody. The total lysate sample is also analyzed in this fashion, and the biotinylation index (eluate signal / total lysate signal) can be interpreted as an indication of the amount of protein translocation.

Expected Results

We predict that pharmacologic inhibition of PI3K and Akt will inhibit insulin-stimulated phosphate transport in both L6 cell culture and mouse soleus muscles. We expect that knock-down of PI3K will also result in inhibition of insulin-stimulated phosphate transport, and that overexpression of constitutively active p110 will have the opposite effect, in L6 cell culture. Furthermore, we would predict that in the case where insulin-stimulated translocation of, for example, PiT-1 is observed, that this effect can be attenuated by pharmacological inhibition of PI3K and Akt.

Limitations and Alternatives

It is possible that the mechanism of action of insulin-stimulated phosphate transport does not act through PI3K. If this turns out to be the case, other downstream effectors of insulin will need to be investigated. With regards to the planned p110 overexpression experiment, the effects of acute insulin stimulation may be very different from those of chronic stimulation, and thus constitutive PI3K activity through p110 may not mimic acute insulin stimulation. As a result, a

negative result here will be difficult to interpret. With respect to the PI3K knock-down experiment, there are several isoforms of PI3K and knocking down one of them may not have the anticipated effect of significantly inhibiting insulin-stimulated phosphate transport.

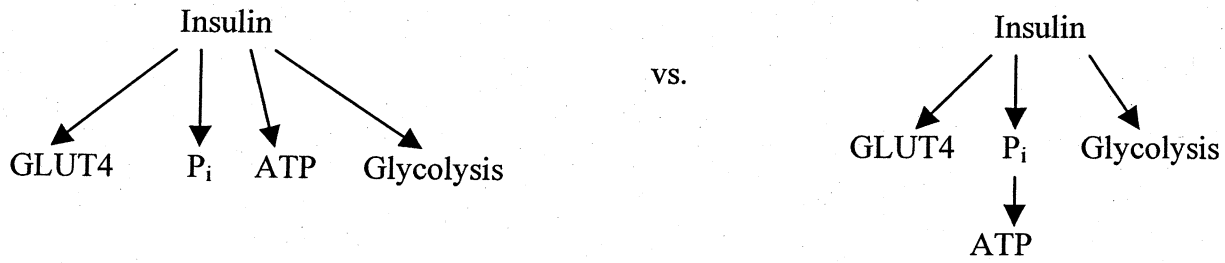
With respect to the transporter assays described above, namely the ELISA and immunofluorescent-based methods, the principal limitation is the availability of commercially available antibodies targeted to the extracellular epitopes of our transporters of interest. Although the surface biotinylation method does not fall prone to the requirement of an extracellularly targeted antibody, all three methods must be sensitive enough to detect changes on the order of 20 – 50%, the order of which we have observed in the L6 system.

One intervention that can improve the sensitivity of all three of these methods is to engineer a commonly used epitope tag (i.e. myc or HA) into an extracellular region of our transporter of interest, and to express this construct in our cell system by transient or stable transfection. Given the commercial availability of specific antibodies to such tags, the ELISA, immunofluorescent, and surface biotinylation methods may be rendered sensitive enough for our detection/quantification needs.

AIM 3: DETERMINE THE REGULATORY ROLES OF INORGANIC PHOSPHATE TRANSPORT ON ATP SYNTHESIS IN SKELETAL MUSCLE.

Rationale

Type 2 diabetes mellitus is a significant and growing global health concern. Insulin resistance is considered to be a key step on the road to this devastating disease. The pathogenetic mechanisms of insulin resistance, however, are still not very well understood. Recent evidence points to the potential involvement of mitochondria in the development of insulin resistance [29]. In a study conducted by our group in 2005 [5], offspring of type 2 DM parents exhibited decreased rates of insulin-stimulated glucose transport, phosphate transport, and ATP synthesis relative to insulin sensitive controls. In light of the fact that P_i is a necessary substrate for ATP synthesis, and that the uptake of the former and the synthesis of the latter are both stimulated by insulin, two scenarios can be imagined as depicted below. In the first, the consequences of insulin stimulation can act in parallel to each other, while in the second, they can act in series with one another.



Hypothesis

We hypothesize that insulin-stimulated phosphate transport may mediate the increase in ATP synthetic rate observed under insulin stimulation. In other words, that insulin-stimulated ATP synthesis in skeletal muscle is effected through an increase in insulin-stimulated intracellular phosphate.

Methods

There are a variety of available techniques for the evaluation of ATP production in biological samples. Two of the more commonly used methods involve HPLC-based and luciferin/luciferase-based techniques. We plan to employ both of these techniques, as necessary, in addition to a third and less common approach which makes us of a phenomenon known as saturation transfer in conjunction with magnetic resonance spectroscopy (MRS).

The HPLC-based method can be used on animal tissues or cultured cells to quantify the amounts of ATP, ADP, AMP, and PCr present. This is a chromatographic technique where a standard mixture containing known amounts of the desired species is eluted from a chromatographic column, and elution time and area under the curve can be determined for each standard. Thus, when the sample is run on this same column and eluted, each peak can be identified by its elution time and quantified by its area under the curve. The principle advantages inherent in this method include the ability to measure multiple species at a time and the very small sample size requirements.

Another commonly used approach to ATP quantification in cultured cells is one invoking the properties of firefly luciferase. In the presence of ATP, firefly luciferase will oxidize luciferin (which has reacted with ATP to form adenyl-luciferin) to oxyluciferin. This oxidation reaction also produces a flash of yellow-green light with a peak emission at 560 nm, the intensity of which is proportional to the amount of substrates present [30]. In a situation where the limiting reagent to this reaction is ATP, the amount of ATP present in a given sample can be determined from the intensity of light emitted from the reaction mixture by comparing this value to a previously determined standard curve of light emitted vs. ATP concentration. There are several variations to this approach. One approach calls for cell lysis and ATP release, followed by addition of luciferin and luciferase to the reaction mixture. Another approach makes use of targeted forms of luciferase that can be transfected into cultured cells and can be used to measure ATP concentrations in more specific cellular locales. For example, luciferase expression vectors have been developed to target luciferase to the mitochondrial matrix, the plasma membrane, and the nucleus. The principal advantages of this method include the ability to measure specific ATP pools, the relative ease of use, and the potential for measuring large numbers of samples in an automated, reproducible fashion.

The principal disadvantage of the two methods described above is that they both measure the absolute concentration at any one given point in time. These methods are not capable of measuring the rate of ATP synthesis. It would be hypothetically possible, using the HPLC and luciferase-based methods, to measure the rate of change of ATP concentration under different treatment conditions by stopping the experiment at different times and measuring ATP concentration. This assessment, however, cannot disentangle the rate of ATP disappearance due to any number of cellular processes, and thus cannot assess the true rate of ATP synthesis, which is the question specifically addressed by our hypothesis.

A third, albeit more complicated and technically difficult method, can be used to specifically address the question of changes in ATP synthetic rate. This method is based on magnetic resonance spectroscopy (MRS) of the ^{31}P nucleus in conjunction with a phenomenon known as saturation transfer. The first step in this technique is to obtain a ^{31}P spectrum of the sample in question. The next step is to electromagnetically pulse the gamma phosphate of ATP in such a manner as to render it magnetically invisible, and then obtain a second spectrum. Since the gamma phosphate of ATP is in exchange with the free P_i pool, there will be reduction in the Pi peak proportional to the amount of ATP synthesized in a given amount of time. Thus, the ATP

synthetic rate can be determined. A more detailed explanation of this technique is provided in the *Background and Significance; ^{31}P MRS and Saturation Transfer* section on page 5 of this prospectus.

Our experimental design will be aimed at characterizing the effects of two specific perturbations on ATP synthesis. The first is that of changes in intracellular P_i, and the second is that of insulin and insulin signaling modulation in the absence of extracellular P_i.

In the first instance, we aim to characterize the effect of a change in intracellular P_i concentration on ATP synthesis. This can be done by lysing cultured cells *in situ* and incubating them in buffers of varying phosphate concentrations. Samples of the medium can then be assayed at different time points for ATP content, either by the HPLC or luciferase-based methods, resulting in a kinetic assessment of ATP concentrations under varying amounts of phosphate.

In addition, we aim to characterize the effects of the insulin signaling pathway on ATP synthesis in the absence of extracellular phosphate, thus disentangling the contributions of insulin signaling from those of insulin-stimulated phosphate uptake. Following treatment with, for example, wortmannin and/or insulin, cultured cells can be lysed and ATP assessed by either the HPLC or luciferase-based methods. Kinetic evaluation in this case may not be possible given the requirement that cells remain intact during pharmacologic perturbation to ensure that cell architecture, and possible transduction pathway function, is preserved. The approach of saturation transfer coupled to ^{31}P MRS can be used here to obtain a kinetic assessment of ATP synthesis in response to perturbation of insulin signaling. ATP synthetic rates can be evaluated before and after treatment with, for example, wortmannin and/or insulin.

Moreover, a more definitive experiment can be carried out to assess the role of insulin stimulated phosphate transport on insulin stimulated ATP synthesis. In the case where the effective transporter from Aim 1 is identified, it can be knocked down in cultured cells by siRNA, and these cells can then be stimulated with insulin in the presence of phosphate. Results from this experiment should indicate which of the two hypothetical models, schematized above, are more likely to be correct.

Expected Results

In the first experiment, where cells are lysed *in situ* and incubated with varying amounts of extracellular, and hence intracellular, P_i, we expect to see that ATP levels will increase with increasing amounts of P_i. In the second experiment, where cells are treated with wortmannin and/or insulin in the absence of extracellular P_i, we expect that there will be no change in the levels of ATP or its rate of synthesis. The third experiment is one where a previously identified and characterized insulin-responsive P_i transporter is knocked-down. In this case, under insulin stimulation in the presence of extracellular P_i, we expect to see a blunting of insulin-stimulated ATP synthesis relative to WT control cells, whereas we expect that there will be no difference in ATP synthesis between these two cell types in conditions where extracellular P_i is absent. Whether or not our predictions are correct, however, the data obtained should be useful in differentiating between the possible scenarios, schematized above, regarding the relationship between insulin-stimulated P_i uptake and ATP synthesis.

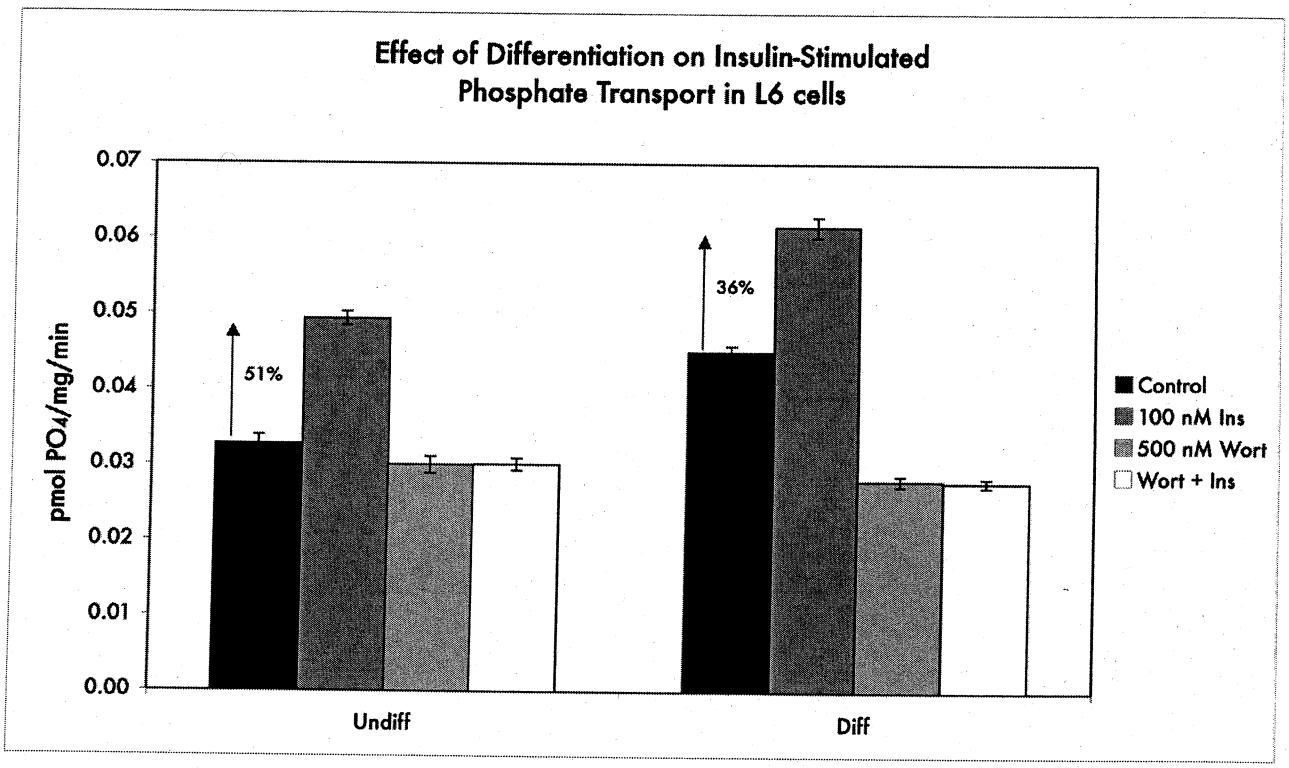
Limitations and Alternatives

In addition to cultured cells, oxygenated mouse soleus muscles can be used wherever possible to corroborate any results obtained. Although we have yet to make use of the HPLC and

luciferase-based methods for ATP quantification, we expect that these methods will be tractable. We expect to encounter the most difficulty in employing the saturation transfer technique in conjunction with ^{31}P MRS. Obtaining an adequate signal to noise ratio, maintaining cell viability during experimentation, and resolving the intracellular P_i signal from other nearby signals are perhaps the greatest challenges. Optimization of the pulse sequences used to saturate and obtain spectra will help in this regard, as well as utilization of a magnetic shift reagent which will help isolate the intracellular P_i peak from adjacent ones.

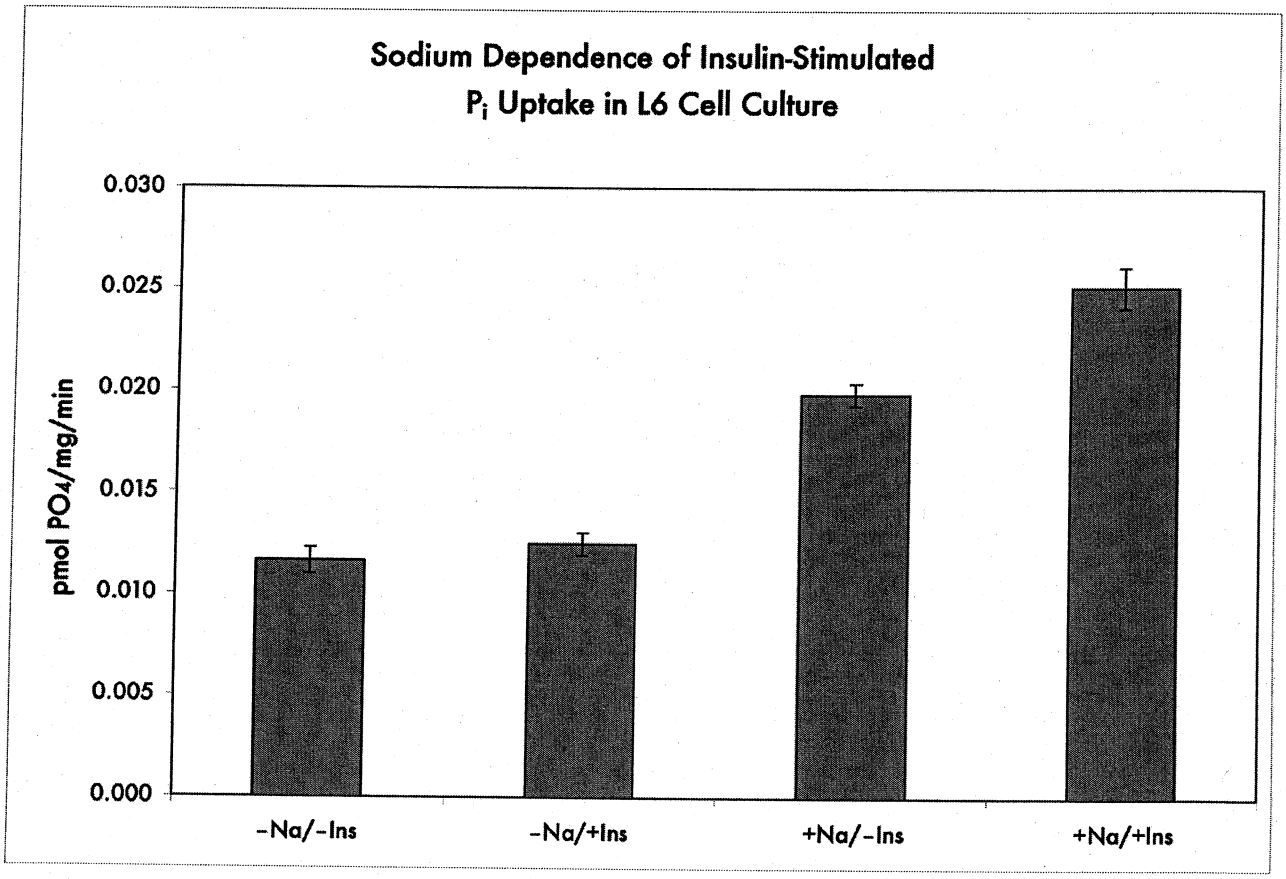
FIGURES

Figure 1.



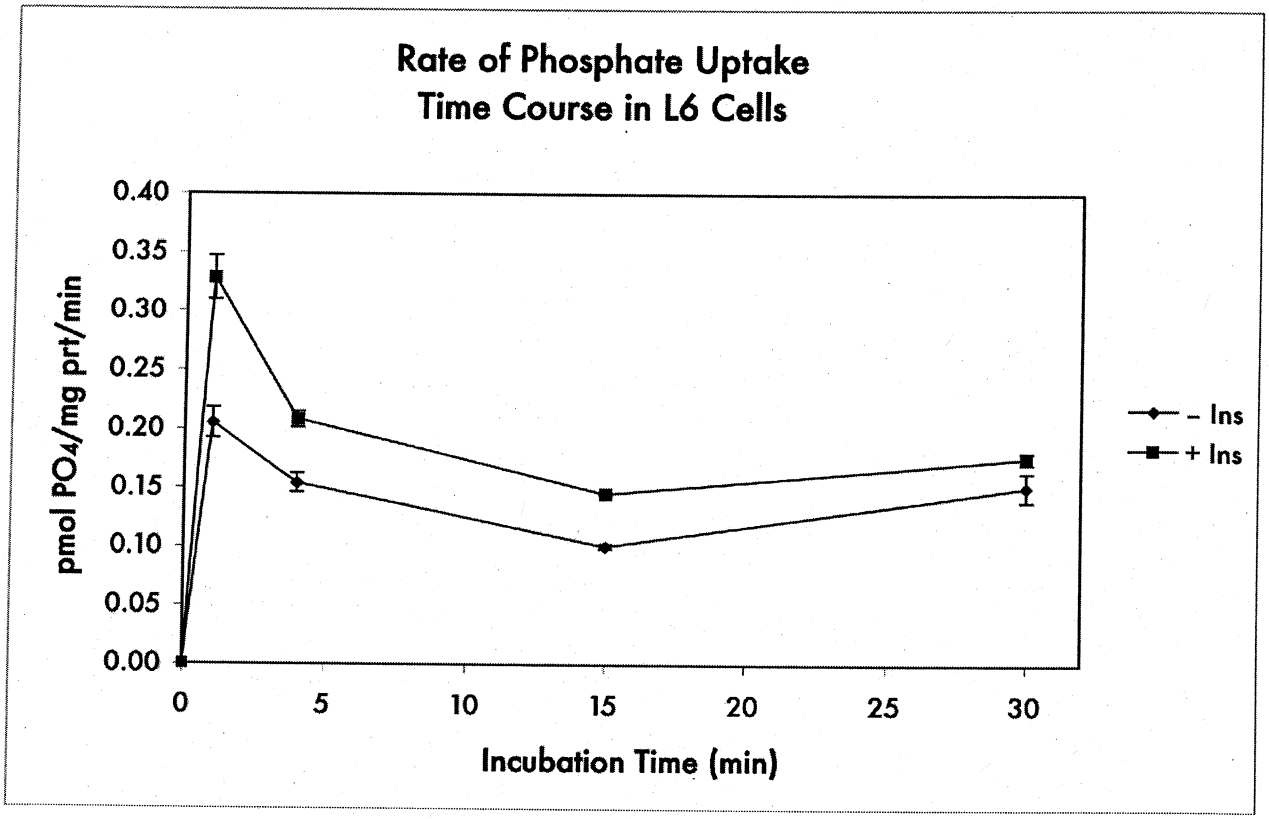
^{32}P uptake assay in undifferentiated and differentiated L6 cell cultures. Black bars; 0.1% EtOH (vehicle). Dark grey bars; 100 nM insulin. Light grey bars; 500 nM wortmannin. White bars; 500 nM wortmannin followed by 100 nM insulin. Wortmannin incubations lasted 20 minutes (where indicated) and were followed by 30 minute incubations with 100 nM insulin (where indicated). Bars represent averages ± SEM with n=6 for each group. Differences in transport rates by insulin stimulation and wortmannin inhibition are statistically significant as evaluated by the t-test with $p < 1 \times 10^{-5}$.

Figure 2.



^{32}P uptake assay in L6 cell cultures under various conditions. Sodium replaced with choline in sodium-free preparations. 100 nM insulin stimulation for 30 minutes where indicated. Bars represent averages ± SEM with n=12 in each group. Evaluation by one-way ANOVA with $p<0.0001$. Post-hoc Tukey's multiple comparison test with 99% CI: $p < 0.001$ for all pairs except –Na / –Ins vs –Na / +Ins.

Figure 3.



^{32}P uptake assay in L6 cell culture. Cells were incubated for varying amounts of time in ^{32}P-containing buffer, and the rate of phosphate uptake is plotted against incubation time. Cells treated with 100 nM insulin for 30 minutes where indicated. Data points represent averages ± SEM with n=6 in each group.

Figure 4.

Western Blot analysis of L6 cell lysates for phospho-Akt with a polyclonal primary antibody directed against Akt phosphorylated on serine residue 473 (Cell Signaling Technologies, Inc.). Cells were pretreated with 1 μM wortmannin where indicated, and with varying concentrations of insulin, as shown.

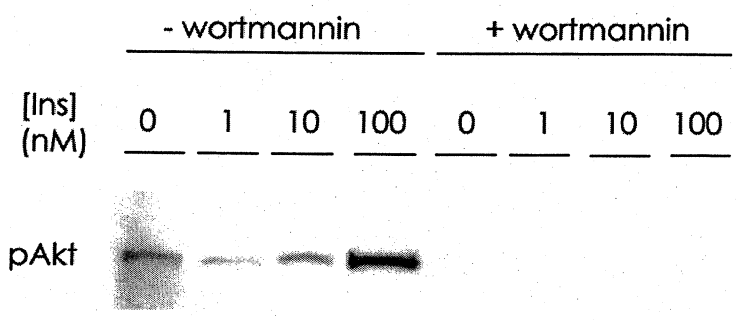


Figure 5.

Western Blot analysis of mouse soleus lysates for phospho-Akt with a polyclonal primary antibody directed against Akt phosphorylated on serine residue 473 (Cell Signaling Technologies, Inc.). Individual muscles were treated with 1 μM wortmannin and 100 nM insulin as indicated, for 60 minutes each.

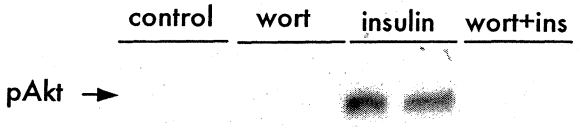
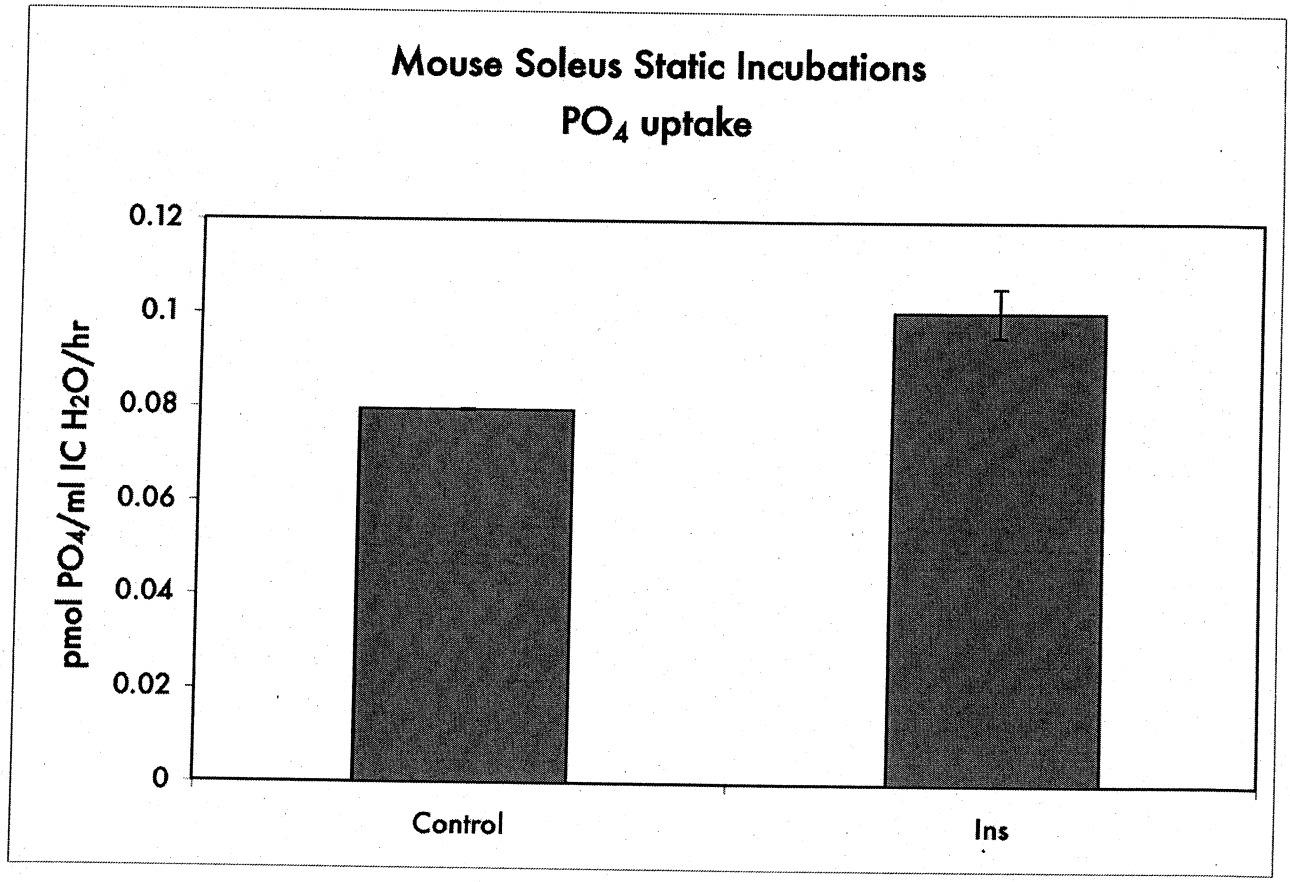


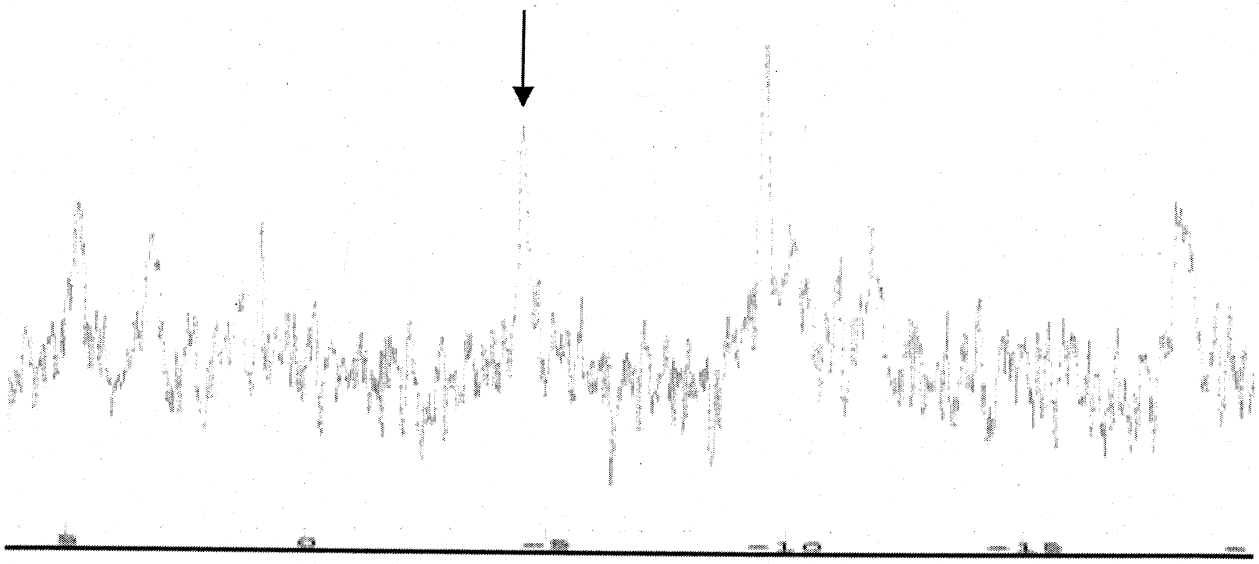
Figure 6.



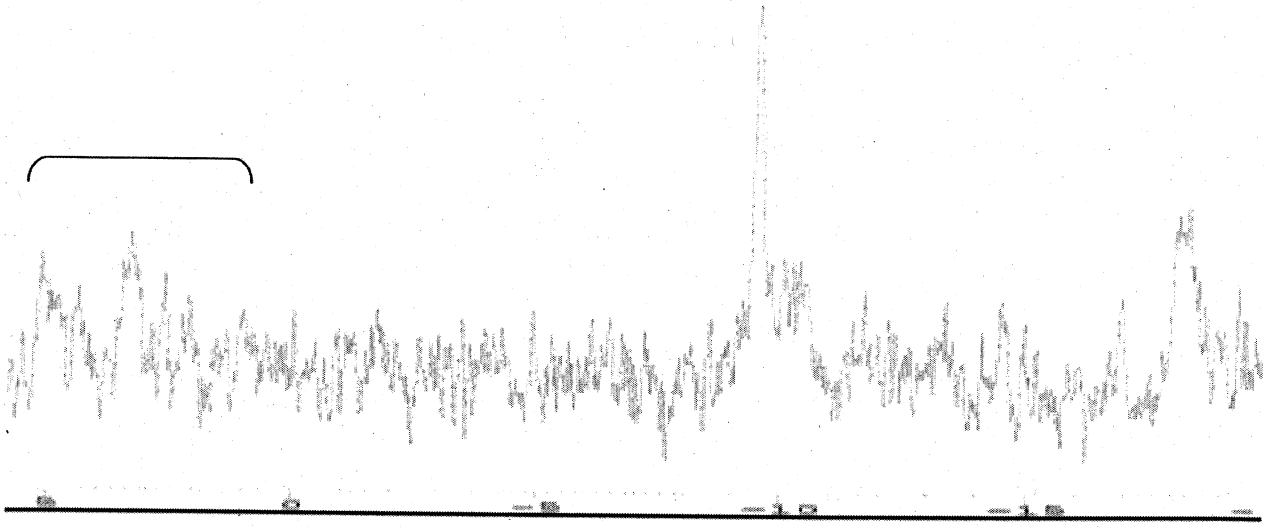
32P uptake assay in mouse soleus muscles. Incubated with 100 nM insulin where indicated. Bars represent averages ± SEM with n=3 in each group. Statistical evaluation by t-test with $p<0.05$.

Figure 7.

A.



B.



Exemplary spectra of ^{31}P MRS saturation transfer experiment. The ATP-γ-P_i is saturated with a pulse centered at -897 Hz (arrow in spectrum A), and a second spectrum, B, is obtained. The intracellular P_i peak resides somewhere in the bracketed region, and some attenuation of signal can be observed.

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